INVESTIGATING THE FLAVOR OF FRESH CALAMONDIN PEEL AND JUICE USING INSTRUMENTAL AND DESCRIPTIVE SENSORY ANALYSIS

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DEDICATION

For my Mom, G-Ma, and all the strong women in my life.

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ABSTRACT

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Calamondin *(Citrus microcarpa)* is a popular citrus fruit in Asia that resembles a small tangerine with a delicate pulp and fresh, lime-like flavors in its peel and juice. Studies on the flavor of calamondin juice and peel are limited and its unique flavor has not been well characterized. The objective of this study was to investigate the flavor composition and sensory properties of fresh immature calamondin juice and peel. A method using solid-phase extraction followed by gas chromatography-mass spectrometry (SPE-GC-MS) analysis was developed for volatile isolation and identification in calamondin juice and peel. The developed method used Lichrolut-EN sorbent and a 95:5 dichloromethane:methanol eluent that was effective for extraction of a wider range of volatiles compared to the most popular method, solid-phase microextraction (SPME)-GC-MS. SPE-GC-MS analysis identified 75 and 101 compounds from the juice and peel, respectively. The total volatile intensity of the peel was more than three times that of the juice. The dominant peel volatiles included limonene (10.53-27.85%), *(Z)*-3-hexenol (4.85-12.51%), linalool (9.40-10.29%), 1-octanol (2.55-2.84%), α-terpineol (4.00-7.80%),

isopiperitenone (1.91%), geraniol (0.79-1.06%), 8-hydroxylinalool (1.20-2.12%), *(E)*-ρ-mentha-2,8-dien-1-ol (0.39-1.61%), and hexadecanoic acid (0.81-1.31%). Dominant juice volatiles included limonene (14.51-14.59%), hexadecanoic acid (3.19-10.88%), 4-hydroxy-benzeneethanol (0.09-7.98%), cryptomeridiol (4.95-5.76%), stearic acid (3.38-3.82%), α-terpineol (2.29-3.76%), *(Z)*-8-hydroxylinalool (0.45-3.58%), α-cadinol (1.23-3.16%), limonen-1,2-diol (0.41-2.85%), linoleic acid (1.36-2.73%), and *(Z)*-3-hexenol (0.17-1.36%). The volatile profiles showed seasonal difference, with fruit harvested in August containing higher concentrations of most volatiles compared to fruit harvested in April. Sensory evaluation was conducted by quantitative descriptive analysis (QDA) where panelists (n=12) used 19 attributes and their intensities (0-10 line scale) to characterize the flavor of calamondin juice and zest. QDA indicated that the aroma of the zest was most intensely characterized by peely, fresh, and fatty notes, with intensities of 6.8, 5.7, and 5.3, respectively. The aroma of the juice was most intensely characterized by juicy, acidic, mandarin, and fresh with intensities of 5.8, 5.7, 5.5, and 5.2, respectively. The flavor (aroma and taste) of the juice was most intensely characterized using the attributes of sourness (8.9), salivating (7.8), astringent (7.5), bitter, juicy (5.6), and fresh (5.2). Results of the chemical and sensory analysis indicated that dominant volatiles identified in the juice and peel corresponded to sensory attributes. This study could be applied towards developing a flavor profile for calamondin.

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CHAPTER I

INTRODUCTION

Calamondin *(Citrus microcarpa)* is a citrus fruit that resembles a small tangerine, with a delicate pulp and a lime-like flavor (Bender, 2014). It is an inter-genetic hybrid between a mandarin orange (*Citrus reticulate*) and kumquat (*Citrus japonica*) member of the Rutaceae family. Calamondin has recently been given the hybrid name of *Citrus x citrofortunella mitis* (Morton, 1987). It is also identified as *Citrus mitis* Blanco, *Citrus microcarpa* Bunge, or *Citrus madurensis* Lour. Other common names for the fruit are calamondin orange, Chinese orange, Panama orange, golden lime, calamansi, or limonsito (Morton, 1987). The oblate fruit can contain up to five seeds and grows to approximately 3 – 4.5 cm in diameter with an aromatic, thin, glossy peel dotted with numerous small oil glands (Morton, 1987). The peel color ranges from a dark to light green in its immature stage, and a yellow to yellow orange when fully mature. The orange colored pulp of the fruit can be separated in six to 10 segments, is juicy, and highly acidic (Morton, 1987).

Although produced commercially in other countries, the potential expansion for calamondin in the United States does not appear great due to the availability of other citrus fruit with identical uses (Nisperos-Carriedo, Baldwin, Moshonas, & Shaw, 1992). However, with the exotic fruit trend growing, there can be a demand for the fruit in certain industries. More recently in Taiwan and the Philippines, a demand for calamondin juice has increased due to its use in the adulteration of a growingly popular fruit known as

shiikuwasha (*Citrus depressa Hayata*; Yamamoto et al., 2012). Shiikuwasha farmers could not keep up with supply of the fruit, so adulteration with calamondin juice, which resembles shiikuwasha juice in color and flavor, became prevalent commercially (Yamamoto et al., 2012).

Calamondin juice is commonly used as a seasoning and condiment in food to accentuate flavors in sauces, fish, beef, and chicken dishes (Chen et al., 2013; Cheong et al., 2012b). The whole fruit can be placed in boiling water to make a fruit tea, with the juice having a similar flavor profile to lemon and lime, that can be used to make beverages, cakes, marmalades, pies, and preserves (Aggie Horticulture, n.d.). Calamondin imparts tart and acidic taste, which is a common characteristic of fruits in the citrus family. Commercially bottled juice is typically combined with an emulsifier such as gum tragacanth, followed by pasteurization and bottled for distribution (Morton, 1987).

To date, the only reports of volatile components of calamondin juice have been evaluated in mature calamondin fruit. Major components identified from solvent extracted juice include terpene hydrocarbons, which contribute to citrus and woody notes, comprising predominantly of limonene, germacrene D, and β-myrcene (Cheong et al., 2012b). Major terpene alcohols identified include linalool and α-terpineol, both important compounds that depending on their amounts present, may determine the organoleptic quality of citrus juice (Cheong et al., 2012b). Linalool imparting a floral, citrus blossom note, is a typical alcohol found in citrus, while α-terpineol is synthesized from linalool through oxidation and cyclization, which can be used to indicate flavor quality in citrus

juice (Cheong et al., 2012b). Other alcohol compounds present: 1-octanol, *(Z)*-3-hexenol, β-elemol, and β-eudesmol are known to impart floral, fresh, fruity, green, and woody notes (Cheong et al., 2012b). Aldehydes reported include decanal, nonanal, octanal, undecanal, and perilla aldehyde, imparting fatty, green, and peel-like notes (Cheong et al., 2012b). Ester compounds identified include octyl acetate, citronellyl acetate, decyl acetate, geranyl acetate, and geranyl propionate, which are known to contribute floral, waxy, green, and fruity notes (Cheong et al., 2012b). Other trace compounds identified in juice of mature fruit include δ-3-carene, α-terpinene, *(Z)*-β-ocimene, dehydro-β-cymene, α-copaene, *(E)*-4,8-dimethyl-1,3,7-nonatriene, ethanol, 4-terpineol, carvacrol, and *(E)*-2-heptenal (Cheong et al., 2012b).

Published volatile composition analysis of calamondin fruit includes isolation methods of solvent extraction, headspace analysis, solid-phase microextraction (SPME), and distillation (Chen et al., 2013; Cheong et al., 2012a; Cheong et al., 2012b; Cuevas-Glory, Sauri-Duch, & Pino 2009; Moshonas & Shaw, 1996; Nisperos-Carriedo et al., 1992; Takeuchi, Ubukata, Hanafusa, Hayashi, & Hashimoto 2005; Yo & Lin, 2004). Extracts were prepared from calamondin peel and juice via liquid-liquid extraction (LLE) with the use of solvents dichloromethane, hexane, and diethyl ether, followed by either filtration or centrifugation to separate solids, then concentrated via nitrogen stream or rotary evaporator (Cheong et al., 2012a; Cheong et al., 2012b; Moshonas et al., 1996; Takeuchi et al., 2005). Headspace analysis has been performed on calamondin juice of mature fruit where juice was prepared from manually squeezed fruits that were not peeled, which may have altered

the true original aroma profile of the juice due to the incorporation of peel oil that may have intensified the flavor and thus detection of other compounds not really in the juice (Nisperos-Carriedo et al., 1992). SPME involves exposing SPME fiber to the headspace of a sample and absorbing volatiles, and then inserting the SPME fiber into the GC for analysis. SPME extraction method has been applied to mature calamondin juice, although one of the reports does not state their sample juice was prepared from peeled fruits, thus altering the true quantitative and qualitative results of their study (Cheong et al., 2012b; Yo & Lee, 2004). A distillation method, with volatile compounds carried in the steam, condensed, and then separated, has been performed on the calamondin peel by steam distillation and hydro-distillation using deionized water for extraction of essential oils (Chen et al., 2013; Cuevas-Glory et al., 2009).

Solid-phase extraction (SPE) is a volatile isolation technique, which involves passing the sample through a packed column or a cartridge filled with a solid phase sorbent, where the solutes are absorbed and then eluted with an organic solvent (Cao et al., 2015). Sorbents used in extraction of flavor compounds include silica gels (polar), activated aluminas (polar), activated carbon (nonpolar), zeolites, and polymers, such as polystyrene, polyacrylilc esters, polydimethylsiloxane (PDMS), and phenolic resins (Dziadas, Nowacka, Jesionowski, & Jeleń 2011). As volatiles in various food matrices range from polar to nonpolar, aromatic and aliphatic, use of the correct sorbent should be considered when extracting targeted compounds (Dziadas et al., 2011). To date, there are no current reports on the use of SPE for the isolation and analysis of calamondin flavor compounds.

Sensory analysis is an approach to provide flavor perception as a link to chemical mechanism. Quantitative descriptive analysis, also known as QDA, is a descriptive analysis method in sensory evaluation based on the principle of a panelist's ability to verbalize perceptions of a sample in a reliable manner (Stone, 1992). The method includes a formal screening and training procedure, development and use of a sensory language, and the scoring of samples on repeated trials to obtain a complete, quantitative description (Stone, 1992). Sensory analysis of the flavor and aroma profile of calamondin is vague, as the only current published sensory evaluation of calamondin is on peel extracts (Cheong et al., 2012a). This sensory evaluation described the peel extract to have attributes of fatty, fruity, green, juicy, mandarin, peely, sweet, and woody (Cheong et al., 2012a). Studies have been limited, and there is still a lack of information on the flavor constituents and sensory attributes of calamondin peel and juice.

Objective of Study

The objective of this study was to establish a flavor profile for immature calamondin (*Citrus microcarpa*) fruit utilizing optimal flavor extraction techniques for volatile qualitative analysis combined with sensory approach.

Specific Aims

1. To evaluate extraction techniques and identify volatile compounds present in newly harvested, immature calamondin peel and juice using solid-phase extraction-gas chromatograph-mass spectrometry (SPE-GC-MS) analysis.

2. To assess sensory attributes of newly harvested, immature calamondin peel and juice by conducting a QDA.

CHAPTER II

REVIEW OF LITERATURE

Calamondin Fruit General Characteristics

Calamondin is believed to be native to China and taken in early times to Indonesia and the Philippines (Morton, 1987). Cultivation of the fruit ranges through China, the Philippines, India, Japan, Hawaii, the West Indies, Central and North America, including California, Texas, and Florida. It has been recorded as being introduced into Florida from Panama in 1899, then quickly became popular in Florida and Texas (Morton, 1987).

The calamondin tree can grow ranging from 6 to 25 ft high, is erect, slender, cylindrical, and develops dense branches beginning close to the ground (Morton, 1987). The single leaflets of the tree are alternate, aromatic, broad-oval, dark-green, glossy on the upper surface, yellowish-green beneath, and 1-3 inches in length (Morton, 1987). The flowers have a rich sweet fragrance, are about 1-inch wide, and have five elliptic-oblong, pure-white petals (Morton, 1987). It is a cold-hardy citrus down to 20°F making it more robust to colder weather temperatures than any other true citrus species, and is moderately drought-tolerant (Aggie Horticulture, n.d.; Morton, 1987). Adaptable to climates and environments, the tree is tolerable in a wide range of soils, from clay-loam in the Philippines to limestone or sand in Florida (Morton, 1987). The trees may be easily grown from its seeds, which are poly-embryonic with three to five embryos each (Morton, 1987).

In the Philippines, calamondin is the principal citrus fruit used primarily for its juice and as a substitute for lemon (Moshonas et al., 1996). In commercial fruit production, calamondin trees are budded on calamondin seedlings (Morton, 1987). Trees can also be grown from seeds or from rooted cuttings and can do well as a tub or container plant in colder regions that commonly do not grow citrus (Aggie Horticulture, n.d.). Potted citrus plants prefer bright light, growing best outdoors in direct sunlight or half shade (Aggie Horticulture, n.d.). Temperatures for optimum growth range between 70°F to 90°F and do not grow well at temperatures below 55°F (Aggie Horticulture, n.d.). Optimum growth requires watering only as needed and a water-soluble fertilizer (Aggie Horticulture, n.d.). Calamondins are harvested by clipping the stems as they become fully colored throughout the year and can take up to a year to ripen into the orange-colored mature stage (Aggie Horticulture, n.d.; Morton, 1987). In Asian countries, the fruit is utilized and best known in its immature stage, which is just before maturation when the peel is colored light green to green.

Peak harvesting season is mid-August through October and in North America, may be most abundant from November to June (Aggie Horticulture, n.d.; Morton, 1987). There are usually four or five flushes (periods of new growth) on a citrus tree each year and calamondin is one of the few citrus trees that can flower and set fruit year-round (Aggie Horticulture, n.d.). Flower and fruit will often bloom at the same time (Aggie Horticulture, n.d.). Calamondin is a prime host for Mediterranean and Caribbean fruit flies resulting in less planting in Florida than before (Morton, 1987). The citrus plant can be attacked by the

same pests and diseases that affect the lemon and lime, including crinkly leaf, exocortis, psorosis, xyloporosis, and tristeza, but is immune to canker and scab (Morton, 1987).

Calamondin has various medicinal applications. The juice can be applied to the scalp after shampooing to eliminate itching and promote hair growth, applied directly onto insect bites to relieve itching and irritation, and can also help clear up *acne vulgaris* and *pruritus vulvae* when applied to the skin (Morton, 1987). The juice has also been taken orally as a cough remedy and an antiphlogistic, and when diluted and drunk warm, serves as a laxative (Morton, 1987). The distilled oil of the leaves serves as a carminative with more potency than peppermint oil (Morton, 1987). There are studies stating top benefits of the juice include: boosting the immune system, lowering inflammation, aiding in weight loss, stimulating growth and repair, detoxification of the body, lowering cholesterol, managing diabetes, and treating respiratory infections (Staughton, 2020).

Other applications for calamondin include the use of its juice to bleach ink stains from fabrics and as an ingredient in cosmetic products to lighten the skin (Morton, 1987). In North America, the fruit is typically grown to the mature, yellow-orange color stage and used as a common ornamental dooryard tree (Morton, 1987). Since the fruit takes nearly a year to ripen, it maintains its ornamental landscape value longer than most citrus (Aggie Horticulture, n.d.).

Flavor and Volatile Composition of Calamondin

Investigation of calamondin flavor and aroma has focused on the volatile profiles of the peel oils and juice of the mature fruit (Cheong et al., 2012a; Cheong et al., 2012b;

Nisperos-Carriedo et al., 1992; Takeuchi et al., 2005; Yo et al. 2004). Peel oil extraction of the mature fruit has been described by the attributes of fatty, fruity, green, juicy, mandarin-like, peel-like, sweet, and woody (Cheong et al., 2012a). Volatiles discovered in the peel consisted of alcohols, aldehydes, esters, and acids. Solvent extracted volatile components are comprised predominantly of monoterpenes (limonene, β-myrcene, β-pinene, α-phellandrene, and sabinene), and sesquiterpenes (elemene, farnesene, and germacrene isomers), all of which have been commonly reported in other citrus fruits (Cheong et al., 2012a). Major terpene alcohols identified are linalool and elemol (Cheong et al., 2012a). Others identified are hexanol, *(Z)*-3-hexenol, and *(E)*-nerolidol which are known to impart floral, green, and fresh notes (Cheong et al., 2012a).

Certain aldehydes are known to exhibit intense citrus aroma including the citral stereoisomers (geranial and neral), which have also been identified in the peel (Cheong et al., 2012a). The citral stereoisomers are known to take part in the aroma of kumquat, which is one parent hybrid of calamondin (Cheong et al., 2012a). Other aldehydes identified are *(E,Z)*-2,4-dodecadienal and *(E,E)*-2,4-dodecadienal that are known for intense green, fatty, and oily notes (Cheong et al., 2012a). Esters identified include methyl *N*-methylanthranilate, a key characteristic mandarin-like volatile and methyl salicylate, known for its green and minty properties (Cheong et al., 2012a). Other esters include geranyl acetate, geranyl propionate, citronellyl acetate, and perillyl acetate, known to impart fresh, fruity, and green notes (Cheong et al., 2012a). Additional compounds reported

are limonene oxide (sweet, citrus note), camphor (woody note), and carvone (dill-like, herbal note; Cheong et al., 2012a).

Major compounds identified in both peel and juice include terpenes, alcohols, aldehydes, and esters. The calamondin peel consists of predominantly monoterpenes, such as limonene, β-myrcene, β-pinene, α-pinene, β-phellandrene, and sabinene, as well as sesquiterpenes such as elemene, farnesene, and germacrene isomers (Cheong et al., 2012a; Cuevas-Glory et al., 2009; Moshonas et al., 1996; Takeuchi et al., 2005; Yo et al., 2004;).

Compounds detected in a fresh juice sample include limonene, germacrene D, and β-myrcene, as well as oxygenated compounds linalool and α-terpineol (Cheong et al., 2012b). Additionally, germacrene D and bicyclogermacrene could be used to indicate the quality and freshness of the peel extracts because they are susceptible to isomerization and oxidation (Cheong et al., 2012a).

Essential oils contained high amounts of limonene and β-myrcene (Chen et al., 2013; Cuevas-Glory et al., 2009; Moshonas et al., 1996). The only published research to date on the leaf oil detected high amounts of linalool, β-pinene, and myrcene (Cuevas-Glory et al., 2009). Major compounds detected have typically been identified in mature calamondin fruits, whereas some research did not specify the maturity of the fruit (Chen et al., 2013; Cheong et al., 2012a; Cheong et al., 2012b; Cuevas-Glory et al., 2009; Moshonas et al., 1996; Nisperos-Carriedo et al., 1992; Takeuchi et al., 2005; Yamamoto et al., 2012; Yo et al., 2004). Fruits may have over a hundred different volatile compounds that may vary according to the fruit's ripening stage (d'Acampora, Dugo, Dugo, & Mondello 2008).

Specification of the fruit's maturity stage in analysis is important as a fruit's flavor profile may differ dramatically. The calamondin fruit is typically used in its immature stage, which has a different flavor profile than the mature fruit.

Research on flavor analysis has found differences in volatiles qualitatively and quantitively in calamondin fruits from different countries (Cheong et al., 2012a; Cheong et al., 2012b; Takeuchi et al., 2005; Yo et al., 2004). Fruits from the Philippines and Taiwan were analyzed and compared, and although botanically the same species, researchers detected quantifiable differences in the basic aroma components, particularly the esters (Yo et al., 2004). In a study that compared calamondin fruits gathered from Malaysia, Philippines, and Vietnam, terpenes were detected as the dominant compounds in the peel regardless of geographical origin (Cheong et al., 2012a; Cheong et al., 2012b). Vietnam peel extract had the highest detectable levels of aldehydes compared to the peel from Malaysia and Philippines (Cheong et al., 2012a). The same study concluded that the Vietnam juice contained up to three times the amount of total volatiles than the juice samples from Malaysia and Philippines, although the volatiles mainly consisted of hydrocarbons that contribute relatively little to aroma (Cheong et al., 2012b). Alternatively, the Philippines calamondin juice had the lowest amount of total volatiles but consisted of the highest concentration of acids, alcohols, and aldehydes (Cheong et al., 2012b). Calamondin juice and peel from the three countries shared the same monoterpene and sesquiterpene profiles (Cheong et al., 2012b). However, few studies have focused on the flavor profiles of calamondin grown in the US.

Flavor Isolation Techniques

Identifying volatiles that represent an accurate flavor profile of calamondin are impacted by volatile isolation and detection techniques. Different volatile isolation methods have been used to extract volatiles from the calamondin peel, juice, and leaves of the plant, which include solvent extraction, headspace analysis, SPME, and distillation (Cheong et al., 2012a; Cheong et al., 2012b; Moshonas et al., 1996; Nisperos-Carriedo et al., 1992; Takeuchi et al., 2005; Yo et al., 2004). These methods have their advantages and disadvantages including certain bias and artifact formation due to heat treatment. In general, organic solvent extraction obtains a more complete profile than any other volatile isolation technique and will also extract non-volatiles such as waxes, pigments, and lipids. SAFE (solvent-assisted flavor evaporation) is generally combined with organic solvent extraction to remove non-volatiles while providing discrimination on high-boiling point volatiles as well as volatiles with very low-boiling points. Thus, the selection of an appropriate precise and accurate extraction method has become a prerequisite in advancing the understanding of proper techniques in volatile extraction.

Although the calamondin fruit is well known in Asia, there are only a few publications investigating its flavor composition. Established flavor volatile isolation methods have been performed on the peel, peel oil, juice, and the leaf oil, including solvent extraction, headspace analysis, SPME, SPME cryofocusing, steam distillation, and hydro-distillation (Chen et al., 2013; Cheong et al., 2012a; Cheong et al., 2012b; Cuevas-Glory et al., 2009; Moshonas et al., 1996; Nisperos-Carriedo et al., 1992; Takeuchi et al., 2005;

Yamamoto et al., 2012; Yo et al., 2004). For example, solvent extraction methods have been performed on the peel, peel oil, and juice using solvents such as dichloromethane, diethyl ether, and hexane (Cheong et al., 2012a; Cheong et al., 2012b; Moshonas et al., 1996; Takeuchi et al., 2005). All solvent extractions were followed by concentration using either rotary evaporator or under a stream of nitrogen (Cheong et al., 2012a; Cheong et al., 2012b; Moshonas et al., 1996). The rarely published method of SPME cryofocusing was carried out on the juice that involved a GC column head (10 cm) dipped into liquid nitrogen to collect volatiles (Yamamoto et al., 2012). It has also been reported that peel and whole fruit were homogenized with deionized water and steam distilled for extraction of essential oils (Chen et al., 2013). The fruit peel and the leaves of the calamondin plant were hydro-distilled for 3 hours in a Clevenger-type apparatus to yield volatile oils (Cuevas-Glory et al., 2009).

Flavor profile analysis consists of utilizing a combination of volatile isolation methods to ensure varied compound extraction. One of these methods includes SPE. It is one of the most widely used methods for extraction of organic compounds from various samples, and is able to extract a wide range of analytes from non-polar to highly polar (Andrade-Eiroa, Canle, Leroy-Cancellieri, & Cerdá 2016a; Andrade-Eiroa, Canle, Leroy-Cancellieri, & Cerdá 2016b). Compared to other common isolation methods such as LLE, SPE requires smaller sample volumes and can extract a broad range of compounds with increased selectivity (Andrade-Eiroa et al., 2016b). Basic SPE procedures consist of first conditioning the solid-phase materials (cartridge containing the sorbent) by passing organic

solvents through the column to increase the effective surface area and reduce interferences. The sample solution is then loaded, followed by washing away undesired components using deionized water or methanol, and then the sorbent is dried with air. After drying the sorbent and possibly removing interferences, the interactions between analytes and solid-phase material are disrupted by flushing small volumes of organic solvents, which leads to desorption of target analytes from the solid phase, also known as elution. The eluted sample is collected and may go through further processes, such as concentration (Andrade-Eiroa et al., 2016a).

The majority of published literature using SPE for flavor isolation is on the analysis of wines, but the methods described can be applied to any liquid food sample, depending on the compound of interest to be isolated (Dziadas & Jeleń, 2010; Dziadas et al., 2011). SPE protocols are not systemized and require a trial-and-error process for optimization, yet they offer a simple and efficient procedure for the isolation of a wide variety of flavor compounds (Andrade-Eiroa et al., 2016b).

Other known flavor isolation techniques include steam distillation (SD), solvent extraction (SE), fractionation of solvent extracts, simultaneous distillation-extraction (SDE), supercritical fluid extraction (SFE), pressurized-fluid extraction, Soxhlet extraction, SAFE, microwave-assisted hydrodistillation (MAHD), direct thermal desorption, (DTD), matrix solid-phase dispersion (MSPD), and methylation (d'Acampora et al., 2008). Distillation and SE methods are the most common and considered to produce extracts that represent the flavor of the food sample, which is not always relevant for the

determination of a characteristic odor profile. A food flavor profile is closely related to the isolation procedure, which should yield a product representative of the sample. The most appropriate way to attain optimum recovery of flavor compounds is using more than one extraction technique (d'Acampora et al., 2008).

Artifacts in Flavor Isolates

Current methods in flavor isolation and extraction methods are consistently modified as certain compounds identified have been shown to not actually be present in the food sample being analyzed. This may be the result of factors such as chemical decomposition of the food product during isolation, enzymatic action by enzyme systems still active in the food system, reaction of individual flavor chemicals during isolation, and artifacts introduced by the isolation equipment or procedure (Jeon, Reineccius, & Thomas 1976). Investigation into these factors affecting chromatographic and flavor profiles has led to the discovery of the formation of artifacts. Elevated temperatures applied during distillation are shown to lead to the formation of artifacts especially with food samples rich in free amino acids and sugars, which can interact through the Maillard reaction and Strecker degradation to form additional and non-genuine compounds (Majcher & Jeleń, 2009). Occurrence of artifacts was more pronounced at higher fractionation temperatures and time causing significant changes in the chromatographic profiles (Rivellino et al., 2013). In a study of extruded potato snacks comparing flavor isolation methods using SPME and SAFE, which requires low temperatures versus SDE which requires high temperature, the formation of the following compounds was found: 2-Furfurylthiol, 2,5-

dimethyl-3-furanthiol, octanal, *(E)*-2-octenal, and nonanal present in the analysis of the SDE method samples was not present in the SPME or SAFE method samples (Majcher & Jeleń, 2009). 2-furfurylthiol is a main contributor to the characteristic aroma of roasted coffee, roasted beef, or toasted bread and 2,5-dimethyl-3-furanthiol, with a strong smell of boiled meat, is formed from furfuryl alcohol, one of the main Maillard reaction products (Majcher & Jeleń, 2009). Although SDE method is one of the most popular extraction techniques for flavor isolation, it should be used with caution as studies have shown that aroma-active compounds such as 2,6,6-trimethyl-1,3-cyclohexadiene-1-carbon acid, a main aroma compound of saffron, and 5,6-dihydro-2,4,6-trimethyl-4H-1,3,5-dithiazine are formed in the course of SDE extraction (Majcher & Jeleń, 2009). The formation of esters or acetals is also possible during continuous distillation (Majcher & Jeleń, 2009). In a study on fungal volatile metabolites, a high amount of oxygenated terpene compounds obtained by SDE extraction were caused by long-term temperature influence on highly unsaturated sesquiterpene hydrocarbons (Majcher & Jeleń, 2009). Other known artifacts that correlate with foods exposed to improper thermal treatment are: hydroxymethylfurfural (HMF), 2-methyl-furane, and furfural (Rivellino et al., 2013).

Artifacts such as octanal, *(E)*-2-octenal, and nonanal are oxidation products of oleic and linoleic acids (Majcher & Jeleń, 2009). Artifacts introduced by the isolation equipment or GC analysis include antifoaming agents, septum bleed, and vacuum grease, which have been attributed to volatile silicone compounds in the chromatographic flavor profile (Jeon et al., 1976). Distilled laboratory water has shown to contribute residuals, as well as

extracting solvents, such as redistilled dichloromethane, which contained cyclohexane, acetone, and chloroform as trace impurities (Jeon et al., 1976). Artifacts present in the chromatograms must be identified as such, because it presents no relevant chemical information about the sample, or a further meticulous sample preparation procedure must be developed (Rivellino et al., 2013).

Aroma-active Compounds Determined by GC-Olfactometry (GC-O) Analysis

Only a small fraction of the large number of volatiles occurring in foods contribute to odor and aroma; therefore, the distinction between odor-active compounds and the whole range of volatiles present in a food product is an important task in flavor analysis (Delahunty, Eyres, & Dufour 2006; van Ruth, 2001). Commonly, odors perceived in nature, foods, and fragrances are complex mixtures of many volatile compounds (Delahunty et al., 2006). Many natural flavor compounds occur as specific chiral isomers, and their odors can be very distinctive and characteristic (d'Acampora et al., 2008). In analysis and identification of compounds in food flavors, it is important to consider that distinct enantiomers may impart different flavors, have distinct degradation pathways, and often be characterized by different biological activities (d'Acampora et al., 2008).

Experience shows many odor-active compounds occur at very low concentrations and their sensory relevance is due to low odor thresholds (van Ruth, 2001). Gas chromatograph-olfactometry (GC-O) uses human assessors to detect and evaluate volatile compounds eluting from a GC separation (Delahunty et al., 2006). For each separated compound that emerges from the GC, a human assessor has the potential to detect the

compound (odor present or not), measure the duration of the odor activity (start to end), describe the quality of odor perceived, and quantify intensity of the odor (Delahunty et al., 2006). GC-O was proposed by Fuller et al. as early as 1964, using expert perfumers as assessors and is shown to be a valuable method for selection of odor-active compounds from complex mixtures (van Ruth, 2001; Delahunty et al., 2006).

Qualitative and quantitative analysis of aroma-active compounds by GC-O depends on the isolation method utilized (van Ruth, 2001). Solvent extraction or distillation methods often yield total extracts of a sample that do not necessarily represent the proportion of compounds that are perceived by a subject when smelling (orthonasal perception) or eating (retronasal perception; Delahunty et al., 2006). Highly volatile compounds may contribute to the top-note of a product and these may be lost during solvent extraction, distillation, and concentration procedures resulting in an extract not representative of the original sample (Delahunty et al., 2006). Highly volatile compounds could also coelute with the solvent peak, making them unperceivable by GC-O (Delahunty et al., 2006). The sample used for GC-O analysis should represent the aroma/odor composition expressed when foods are eaten or smelled (van Ruth, 2001). It may be challenging to replicate certain food products due to generation of specific volatile compounds by endogenous enzymes in the food caused by the breakdown of the food matrix through mastication, as well as hydration and dilution by saliva (van Ruth, 2001). It is also possible that subthreshold addition or synergy can occur between volatile compounds, so compounds present at concentrations that are below threshold, or possess

no odor activity when assessed individually, may in fact contribute or possess odor activity when mixed (Delahunty et al., 2006).

The conditions of samples for volatile analysis requires careful consideration. Long storage periods of samples should be avoided due to liable volatile compounds that occur in low concentrations and unstable volatiles that readily decompose in heated injector blocks, forming artifacts (van Ruth, 2001). Chromatographic behavior of compounds varies with the stationary phases of the GC column, and can affect GC-O data (van Ruth, 2001). The use of two stationary phases has been recommended to improve resolution of compounds of interest and to improve the strength of identification of odorants (Delahunty et al., 2006). Using two stationary phases may alter elution order and allows for evaluation of cross-adaptation, where a strong odor may affect the odor intensity of a close eluting odor (Delahunty et al., 2006).

Methods have been developed to collect and process GC-O data and estimate the sensory contribution of single odor-active compounds. They can be classified into four categories: dilution analysis, detection frequency, posterior intensity, and time-intensity (van Ruth, 2001). Dilution analysis methods produce potency values based on stepwise dilution to threshold, e.g. combined hedonic response measurement (Charm-Analysis) and aroma extraction dilution analysis (AEDA; van Ruth, 2001). This technique was developed by two different research groups to simplify the method used for determining a unit of odor intensity, aiming to determine relative odor potency of compounds present in an extract (van Ruth, 2001). In Charm-Analysis, dilutions are presented in randomized order to avoid

bias introduced by knowledge of the samples (van Ruth, 2001). In AEDA analysis, the dilution factor (FD value) is the last dilution at which an odor-active compound is detected (van Ruth, 2001). Detection frequency methods record detected odors over a group of assessors: the number of assessors detecting an odor (detection frequency) are used as an estimate of the compound/odor's intensity (van Ruth, 2001). The number of assessors perceiving odor-active compounds were shown to relate very well to the intensity of an odor-active compound, recorded after elution from the column (van Ruth, 2001). Posterior intensity methods provide estimates of perceived intensity, which are recorded after a peak has eluted (van Ruth, 2001). This method involves recording odor intensity on a scale after a peak has eluted from the column (van Ruth, 2001). Time-intensity methods establish estimates of perceived intensity recorded simultaneously with elution of chromatographic peak, also known as the Osme method. This method was developed by McDaniel et al. in 1990 (van Ruth, 2001). Trained assessors directly record intensity, duration of each odor active compound detected at the sniff port, and describe odors perceived (van Ruth, 2001).

Results of GC-O analysis can be affected by the state of the human assessors, causing experimental bias. Decreased alertness may occur due to a sample having only a small number of compounds that can be perceived, compounds that show a low odor intensity, when a stimulus is brief, when a session is long and when assessors are not motivated (van Ruth, 2001). Proper assessment of samples may also be affected due to varying inter-stimulus intervals causing assessors to make decisions very rapidly (van Ruth, 2001). Error of anticipation may occur when an assessor responds in anticipation of

an odor occurring, as in detection frequency and direct-intensity measures (Delahunty et al., 2006). The same extract is likely to be evaluated many times by an assessor, and retention times where odor-active compounds elute will be learned (Delahunty et al., 2006). Many authors showed large variability within and between assessors, so a group of assessors is a prerequisite for reliable GC-O analysis (van Ruth, 2001).

Common objectives of GC-O analysis include characterizing an aroma profile of a sample/extract, quantifying the relative importance of aroma-active compounds and their significance within the sample, identifying compounds responsible for foreign taints or off-flavors and determining their cause, investigations into odor thresholds of unidentified compounds, and discovery of new potent odorants present at trace levels (Delahunty et al., 2006). For example, GC-O led to the discovery of 1-ρ-menthene-8-thiol as an impact odorant of grapefruit juice (Delahunty et al., 2006). This compound is typically present at sub-ppb levels but has an extremely low odor threshold (Delahunty et al., 2006). To correlate chemical composition with sensory data, GC-O is the appropriate lab technique used to identify aroma-active compounds and link to sensory properties.

Quantitative Descriptive Analysis

Sensory analysis, also known as quantitative descriptive analysis (QDA), is the preferred technique to relate information of aroma volatiles to sensory perception (Ren et al., 2015). Descriptive sensory analysis is one of the most sophisticated and informative methodologies to the sensory professional. It is a total sensory description, taking into account all sensations that are perceived, including product's appearance, aroma, flavor,

and texture attributes. The procedure of QDA includes a screening and training procedure, a sensory language development, an assessment of product scoring, and result analysis all based on a panelist's ability (Qiu & Wang, 2015). A descriptive language developed by selected subjects to describe attributes of a product is essential for the successful outcome of a sensory test. However, words only provide a common basis for the scoring of an array of products; it is a scale used for quantifying responses to stimuli. A descriptive test yields a large sensory database, permitting a wide range of statistical analyses.

Sensory evaluation has been conducted on the peel extract of mature calamondin fruit by experienced flavorists (Cheong et al., 2012a). The attributes fatty, fruity, green, juicy, mandarin-like, peel-like, sweet, and woody were established (Cheong et al., 2012a). The sensory evaluation concluded that the peel extract ranked highest for peel-like notes, followed by juicy and mandarin-like notes, with woody being the weakest (Cheong et al., 2012a). Other attributes used to characterize the peel extract are citrus-like, petitgrain-like (essential oil extracted from the leaves and green twigs of the bitter orange tree), citrus, orange-like, sweet, floral, and fruity (Takeuchi et al., 2005). Attributes used to describe the juice extract are sweet, woody, floral, lilac-like, petitgrain-like, acidic, and astringent (Cheong et al., 2012b; Takeuchi et al., 2005).

Sensory evaluation of peel extract samples concluded that different extraction techniques can significantly affect the chemical composition and odor profiles of the volatile extract (Sun et al., 2014). Calamondin is more commonly used in its green colored immature stage in which the flesh of the fruit is characterized by fresh, green, and juicy

notes, exhibiting a remarkably different flavor and aroma than the yellow-orange colored mature fruit. To date, there are no publications on sensory evaluation of calamondin fruit in its more familiar immature stage.

CHAPTER III

METHODOLOGY

Reagents and Standards

The following standards were prepared in methanol at 20 ppm each: ρ-cymene, octanal, hexanol, methyl octanoate, octanoic acid, methyl nonanoate, decanal, linalool, terpinen-4-ol, farnesene, menthol, *(Z)*-verbenol, α-humulene, citral, α-terpineol, *(Z)*-3,7-dimethyl-2,6-octadien-1-yl acetate, carvone, geranyl acetate, 1-decanol, β-citronellol, geraniol, guaiacol, hexanoic acid, β-ionone, perillyl alcohol, nerolidol, cedrol, 5-hydroxymethyl-2-furaldehyde, phytol, and *(E)*-cinnamic acid. Standards were injected into the GC-MS using the same parameters of analysis for SPE volatiles.

Sample of Calamondin

The immature fruit of calamondin (*Citrus microcarpa)* used in this study was purchased from the commercial citrus grove grower Citri-Care Inc. (Orosi, California). The samples were harvested in the green mature stage, with characteristics of green to dark green colored peel and 3 to 5 cm in diameter. Fruits from harvest dates February 20, April 19, and August 14, 2018 were used for method development and fruits from harvest dates April 30 and August 30, 2018 were used for flavor analysis. Approximately three to five pounds of fruit were shipped within 2 days of harvesting and were packed in plastic containers along with ice packs. The fruits were stored in an individual cooler with ice

packs changed daily. For each experiment, the fruit was used within 3 to 7 days post harvesting. The peel was separated by hand and fruit juice manually hand-squeezed from peeled fruit. Fruit juice was filtered using a stainless-steel mesh strainer to separate the pulp and seeds. Zest was prepared by raking a citrus zester kitchen tool along the colored peel of the fruit, avoiding the pith, which produced fine, thread-like strips.

ºBrix, pH, and TA

Fruits harvested on April 30 and August 30, 2018 were hand peeled and manually squeezed. The crude juice was then filtered using Fisherbrand™ grade P8 Fluted Qualitative filter paper (porosity: coarse, flow rate: fast). Total soluble solids (TSS) content expressed as ºBrix was determined with the use of a refractometer (Atago PAL). The pH was measured with a pH meter (pH glass electrode, Metrohm). Measurement of titratability acidity (TA) was carried out with a titrator (Metrohm 888 Titrando) by titrating 1 mL of filtered juice with standardized 0.1 N NaOH. TA was expressed as citric acid (% citric acid/L of juice). All of these measurements were taken according to manufacturers' instruction.

Volatile Instrumental Analysis

Solid-Phase Microextraction

A SPME fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm film thickness) was used for volatile extraction. 0.1 g of zest was placed into a 20 mL screw top, clear glass, headspace vial, followed by a 1-min nitrogen flush then immediately sealed with a stainless-steel screw cap with silicone

septum. Separately, 3 mL of juice was placed into a 20 mL screw top, clear glass, headspace vial, followed by a 1-min nitrogen flush then immediately sealed with a stainless-steel screw cap with silicone septum. SPME fiber was preconditioned for 5 min at 200 °C. Each sample was incubated for 15 min at 40 °C in agitator (speed: 250 rpm) followed by sample extraction for 20 min.

Samples were directly introduced to the GC column via splitless injection. Sampling (desorb) time was 1 min. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The temperature of the GC injector was 250.0 °C. Oven temperature program: 40.0 °C (1 min. hold) to 230.0 °C at 5.0 °C/min (5 min. hold), giving a total run time of 44 min. The mass spectrometer was operated in EI mode with an ionization voltage of 70 eV. The temperatures of the ion source and interface were 200.0 °C and 230.0 °C, respectively. The mass scan range was *m/z* 30 to *m/z* 400. After each sample injection, the SPME fiber was conditioned for 3 min to clean-up. All samples were prepared and analyzed in triplicate.

Solid-Phase Extraction

SPE was performed using a divinylbenzene sorbent, LiChrolut EN (Merck, Germany), to isolate and extract volatile compounds. Calamondin peel sample was prepared from 100 g of peel blended with 250 – 300 mL deionized water for 30 sec in a commercial laboratory blender (Waring). The homogenized mixture was then poured into centrifuge tubes and centrifuged at 4000 RPM for 10 min to separate the solid particles.

After centrifuging, the aqueous layer was filtered using Fisherbrand™ grade P8 Fluted Qualitative filter paper (porosity: coarse, flow rate: fast), yielding 80 – 100 mL sample.

Calamondin juice sample was poured into centrifuge tubes and centrifuged at 4000 RPM for 10 min to separate the solid particles. After centrifuging, the aqueous layer was filtered using Fisherbrand™ grade P8 Fluted Qualitative filter paper (porosity: coarse, flow rate: fast).

The following solvents were used for elution of analytes from the LiChrolut-EN sorbent to determine which solvent presents optimal volatile extraction: 100% methanol and dichloromethane:methanol ratios of 95:5, 90:10, 85:15, and 75:25.

LiChrolut-EN sorbents were preconditioned with 5 mL of solvent, followed by 10 mL of deionized water. Sample was then loaded onto the sorbent: 80 – 100 mL of peel sample or 50 mL of juice sample. After sample loading, the sorbent was washed with 10 mL deionized water, then dried by slowly pushing approximately 60 mL air through the sorbent column with a syringe. Samples were eluted with 1 mL or 30 mL of corresponding preconditioning solvent.

Elution samples of 1 mL were collected directly into a 2 mL crimp top clear glass vial then crimp sealed with an 11 mm crimp cap with septum. Elution samples of 30 mL were collected in a 250 mL Erlenmeyer flask and dried over anhydrous sodium sulfate. The sample solution was then poured into a round bottom flask and concentrated to approximately 1 mL by distillation using a Vigreux column with the jacketed beaker set at 44.0 °C and the condenser set at 10.0 °C. The 1 mL concentration was filtered through a

Fisherbrand™ 13mm non-sterile, solvent resistant, PTFE syringe filter (0.20µm) into a 2 mL crimp top clear glass vial then crimp sealed with an 11mm crimp cap with septum. Samples were prepared in triplicate and injected into the GC-MS for analysis.

Samples (1 µL) were directly introduced to the column via splitless injection. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The temperature of the GC injector was 250.0 °C. Oven temperature program: 40.0 °C (1 min. hold) to 230.0 °C at 5.0 °C/min (15 min hold), giving a total run time of 54 min. The mass spectrometer was operated in EI mode with an ionization voltage of 70eV. The temperatures of the ion source and interface were 200.0 °C and 230.0 °C, respectively. The mass scan range was *m/z* 30 to *m/z* 400.

GC-MS and Data Post Run

Extracted samples were analyzed using a Shimadzu GC-2010 Plus gas chromatograph (GC) with a Shimadzu QP2020 mass spectrometer (MS). The GC column used was a ZB Wax column (30 m x 0.25 mm x 0.25 µm, Restek). Compound identification was achieved by NIST library GC software, calculating linear retention index (LRI), comparing to retention index from libraries or publications, as well as data comparison to analysis of chemical standards.

Quantitative Descriptive Analysis

Human Participants Approval

Approval for the QDA study was obtained from the Institutional Review Board of Texas Woman's University (TWU) – Denton Center (see Appendices A & B). QDA was conducted at TWU's sensory lab. Panelists consisting of male and female participants were recruited from students and staff at TWU. Training of the panel occurred over eight, 2-hour long sessions consisting of tasting and smelling reference solutions to gain familiarization with the attributes.

QDA References

References used are: Fresh, Fatty, Green, Peely, Waxy, Fruity, Juicy, Lime, Piney, Floral, Woody, Mandarin, Grapefruit, Acidic, and Sweet, which were prepared in a taste base solution of 5% sucrose and 0.6% citric acid in deionized water. Chemicals for each descriptor and their dose is displayed in Table 1.

Table 1

Descriptor standard stock solution preparation

Descriptors	**References**		**Stock Solution (25 mL)**	**Preparation (100 mL DI water)**
Fresh	acetaldehyde	natural, ≥99%, FG, Sigma-Aldrich	2% (0.5µl)	0.05%-50 µl
Fatty	octanal	99%, Sigma-Aldrich	2% (0.5µl)	0.05%-50 µl
Green	cis-3-hexenol	natural, ≥98%, FCC, FG, Sigma-Aldrich	3% (0.75µl)	0.3%-300µl

Peely	octanal	99%, Sigma-Aldrich	2% (0.5µl)	0.03%-30µl
	decanal	≥98% (GC), liquid, Sigma-Aldrich	2% (0.5µl)	0.05%-50µl
Waxy	dodecanal	92%, Sigma-Aldrich	2% (0.05g)	0.1%-100µl
Fruity	ethyl butyrate	≥98%, FCC, FG, Sigma-Aldrich	3% (0.75µl)	0.05%-50 µl
Juicy	orange oil	100%, NOW Essential Oils	10% (2.5µl)	0.0015%-0.15µl
	limonene	97%, Sigma-Aldrich	100%	0.025%-25µl
	ethyl butyrate	≥98%, FCC, FG, Sigma-Aldrich	100%	0.00025%-0.25µl
	acetaldehyde	natural, ≥99%, FG, Sigma-Aldrich	2% (0.5µl)	0.025%-25µl
Lime	citral	95%, Sigma-Aldrich	3% (0.75µl)	0.04%-40µl
	limonene	97%, Sigma-Aldrich	9% (2.25µl)	0.7%-70µl
	eucalyptol	natural, ≥99%, FCC, FG, Sigma-Aldrich	3% (0.75µl)	0.0004%-0.4µl
Piney	pine needle essential oil	100%, NOW Essential Oils	10% (2.5µl)	0.25%-250µl

Floral	orange blossom std	0.1%, Firmenich	orange blossom std	Standard (150µl) was vortexed with 850µl propylene glycol until homogenous, followed by mixing with 250 mL taste base solution. This mixture was then diluted 1:5 in DI water
Woody	cedrol	≥99.0% (GC), Sigma-Aldrich	5% (0.125g)	0.3%-300µl
Mandarin	sinensal fraction	20%, Florida Chemical Company	10% (2.5µl)	0.15%-150µl
Grapefruit	nookatone	≥98%, FG, Sigma-Aldrich	3% (0.075g)	0.02%-20µl
Acidic	acetic acid	natural, ≥99.5%, FG, Sigma-Aldrich	see preparation	0.005% (5µl) in 100 mL DI water
Sweet	vanillin	natural, ≥97%, FCC, FG, Sigma-Aldrich	2% (0.5g)	0.001%-1 µl

Note. 25 mL of stock solution was prepared for each reference. Each taste solution was prepared in 100 mL deionized water.

Sample Preparation

Samples were served at room temperature in 2 oz plastic portion cups covered with a plastic lid and prepared within 30 min of conducting sensory analysis. The following samples were evaluated: zest for smelling, 100% juice for smelling, and diluted juice with deionized water (1:2) for tasting. Zest sample was prepared by raking a citrus zester kitchen tool along the colored peel of the fruit, avoiding the pith, which produced fine, thread-like

strips that were placed immediately into the plastic cup and sealed with the lid to avoid oxidation. The juice sample was filtered using a stainless-steel mesh strainer to separate the pulp and seeds. The diluted juice sample was made by diluting the 100% juice sample with deionized water in a 1:2 ratio.

Sensory Evaluation

Panelists were given paper ballots (see Appendix C) to complete during the sensory evaluation. A ballot was given for each sample (Zest-Smell, Juice (without dilution)-Smell, Juice (1:2 dilution)-Taste). The intensity of each attribute was evaluated across the fruit samples on an unstructured, 0-10 cm line scale with 0 representing *no intensity* and 10 representing *extremely high intensity*. The numbers 0 through 3represents *low* intensity, 4 through 6 represents *medium* intensity, and 7 through 10 represents *high* intensity. Each ballot had a comments section to complete if the panelist desired to include additional comments about the attributes.

The demographic information section (see Appendix D) questioned the frequency of citrus fruit/citrus juice purchasing and consumption, the type of citrus fruit/citrus juice consumed, and preference of type of citrus product (i.e. fresh (raw), juice, canned). Panelists were also asked to indicate their age group: <25, 25-35, 36-45, 46-55, 56-65, >65. Testing was conducted in isolated booths illuminated with incandescent lighting. Panelists rinsed between samples with bottled spring water as well as ate plain saltine crackers for palette cleansing. All samples were evaluated in triplicate.

Statistical Analysis

All data were collected and submitted for statistical analysis. GC-MS data was calculated as % peak area. QDA data verification analysis was performed using F-values via Analysis of variance (ANOVA). Mean score and standard deviation were calculated. ANOVA was performed to compare each descriptor by samples. Tukey's Honest Significant Difference (HSD) test was performed for the pairwise comparisons with $\alpha = 0.05$. Statistical analyses were performed using SPSS version 19. Principal component analysis (PCA) was used to find underlying relationships between the two types of data and analyzed using XLSTAT 2015.

CHAPTER IV

RESULTS

ºBrix, pH, and Titratable Acidity

Calamondin fruit in the immature stage is displayed in Figure 1, which includes samples measuring approximately 3 cm in diameter. This is a typical size of the fruit that ranges from 3 – 4.5 cm in diameter. ºBrix, pH, and titratable acidity (TA) values of calamondin juice were measured and the results are shown in Table 2. The ºBrix values varied at a percent difference of 25.7% with 7.8 ºBrix for harvest date April 30, 2018 and 10.1 ºBrix for harvest date August 30, 2018. ºBrix is a measurement of total soluble solids, including organic acids and soluble pectin, so the difference in the ºBrix values may be attributable to the influence of harvesting time effecting fructose and other soluble solid content. The pH values of the calamondin juice from harvest dates April 30 and August 30, 2018 varied at a percentage difference of 25.3% being 2.21 and 2.85, respectively. TA expressed as citric acid (% citric acid/kg of juice) was measured from harvest date August 30, 2018 at 8.72%. TA was not measured from harvest date April 30, 2018 due to lack of appropriate lab equipment at that time.

Figure 1. Immature calamondin fruit

Table 2

Values of ºBrix, pH, and titratable acidity of calamondin juice

Harvest date	ºBrix	pH	Titratable acidity (g/L citric acid)
4-30-2018	7.8±0.06	2.21±0.00	n/a
8-30-2018	10.1±0.00	2.85±0.03	8.72±0.22

Volatile Isolation Method Development

To develop an ideal volatile isolation method, several tests were conducted on the juice and peel extract of immature calamondin. These tests consisted of comparing the volatile analysis results of SPME and SPE, which used different types of solvents and different volumes of each solvent for SPE elution.

SPME-GC-MS

SPME-GC-MS analysis was performed on the calamondin juice and zest. The identified volatiles in calamondin juice are displayed in Figure 2 and Table 3. A total of 123 volatiles were identified in the juice. The juice consisted of 10 esters, 11 aldehydes, 74 terpenes, 13 alcohols, 7 ketones, 2 acids, 2 furans, and 2 carbohydrates. The major volatiles identified in the juice included limonene (30.81 – 62.11%), β-pinene (0.43 – 4.95%), germacrene D (0.32 – 3.34%), ethanol (2.49%), β-myrcene (1.84 – 2.21%), δ-cadinene

(1.73%), and 4,8-dimethyl-1,3,7-nonatriene (1.54 – 1.66%), accounting for 42.97 – 70.46% of the total volatiles.

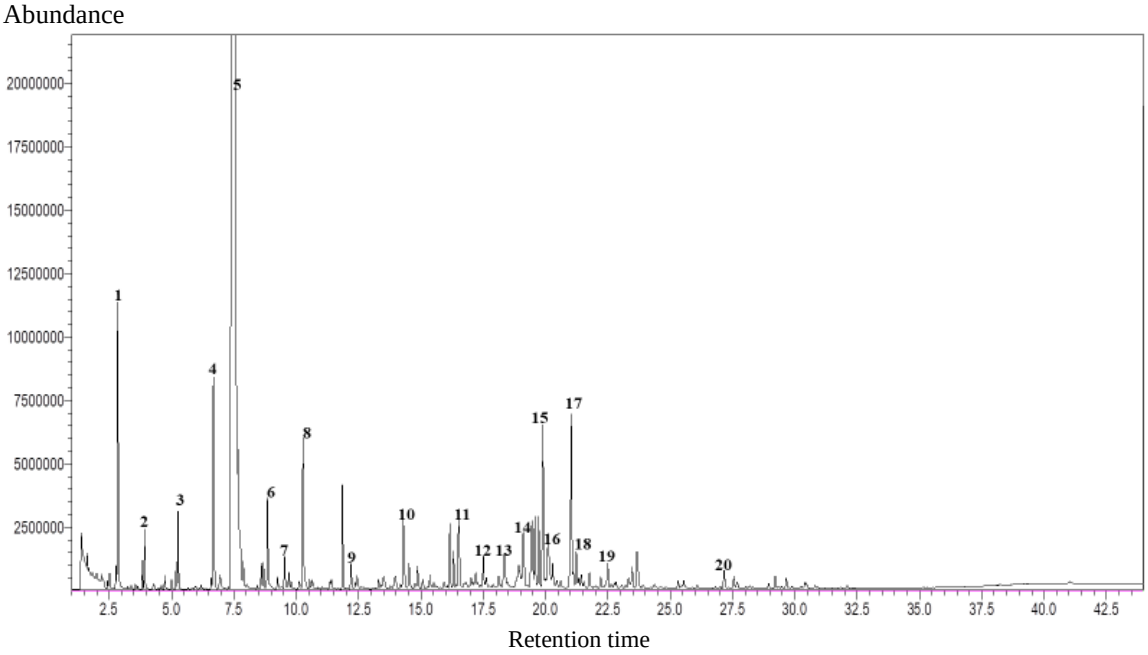

Figure 2. SPME-GC-MS extraction of calamondin juice.

Key: (1) ethanol (2) α-pinene (3) β-pinene (4) β-myrcene (5) limonene (6) *(E)*-β-ocimene (7) octanal (8) 4,8-dimethyl-1,3,7-nonatriene (9) (*Z*)-3-hexenol (10) δ-elemene (11) 1-octanol (12) terpinen-4-ol (13) γ-elemene (14) β-farnesene (15) β-cadinene (16) carvone (17) δ-cadinene (18) decanol (19) germacrene D (20) junenol.

Table 3

Volatile compounds with SPME from calamondin juice using polar column ZB Wax column (30 m x 0.25 mm x 0.25 µm).

RT (min)	LRI	Compound Name	CAS #	April Area %	August Area %	ID Criteria
1.61	**721**	**Acetaldehyde**	75-07-0	-	0.37	MS, RI
2.43	**868**	**Ethyl acetate**	141-78-6	-	0.06	MS, RI
2.69	**914**	**Isovaleric aldehyde**	590-86-3	-	0.04	MS, RI
2.77	**921**	**Isopropyl alcohol**	67-63-0	-	0.16	MS, RI
2.84	**927**	**Ethanol**	64-17-5	-	2.49	MS, RI
3.24	**961**	**2,4-Dimethylfuran**	3710-43-8	-	0.02	MS, RI
3.55	**987**	**Isopropenyl methyl ketone**	814-78-8	-	0.04	MS, RI
3.92	**1013**	**α-Pinene**	80-56-8	-	0.41	MS, RI
4.28	**1034**	**2-Methyl-3-buten-2-ol**	115-18-4	-	0.08	MS, RI
4.47	**1045**	**α-Fenchene**	471-84-1	-	0.01	MS, RI
4.59	**1052**	**Camphene**	79-92-5	-	0.02	MS, RI
4.72	**1060**	**Isopropenyl ethyl ketone**	25044-01-3	-	0.10	MS, RI
4.84	**1066**	**Butyl acetate**	123-86-4	-	0.01	MS, RI
5.00	**1075**	**Hexanal**	66-25-1	-	0.08	MS, RI
5.26	**1091**	**β-Pinene**	127-91-3	4.95	0.43	MS, RI
5.67	**1111**	**Dehydrosabinene**	36262-09-6	-	0.01	MS, RI
5.82	**1118**	**Ethylbenzene**	100-41-4	-	0.01	MS, RI
5.93	**1123**	***(E)*-3-Penten-2-one**	3102-33-8	-	0.04	MS, RI
6.14	**1132**	***ρ*-Xylene**	106-42-3	-	0.02	MS, RI
6.19	**1134**	**3-Carene**	13466-78-9	-	0.04	MS, RI
6.38	**1143**	**1-Butanol**	71-36-3	-	0.01	MS, RI
6.59	**1152**	**α-Phellandrene**	99-83-2	-	0.10	MS, RI
6.68	**1156**	**β-Myrcene**	123-35-3	2.21	1.84	MS, RI
6.95	**1168**	**4-Carene**	29050-33-7	-	0.20	MS, RI
7.20	**1179**	**2-Heptanone**	110-43-0	-	0.00	MS, RI
7.29	**1183**	**Heptanal**	111-71-7	-	0.02	MS, RI

7.57	**1196**	**Limonene**	138-86-3	30.81	62.11	MS, RI, S
7.67	**1200**	**β-Phellandrene**	555-10-2	0.51	0.93	MS, RI
8.02	**1214**	***(E)*-2-Hexenal**	6728-26-3	-	0.03	MS, RI
8.35	**1227**	**2-Pentylfuran**	3777-69-3	-	0.00	MS, RI
8.45	**1231**	***(E)*-β-Ocimene**	3779-61-1	0.02	0.03	MS, RI
8.61	**1238**	**γ-Terpinene**	99-85-4	0.26	0.20	MS, RI
8.89	**1249**	***(Z)*-β-Ocimene**	3338-55-4	1.51	0.83	MS, RI
9.26	**1264**	**ρ-Cymene**	99-87-6	0.05	0.11	MS, RI
9.52	**1274**	**α-Terpinolene**	586-62-9	0.28	0.30	MS, RI
9.70	**1282**	**Acetoin**	513-86-0	-	0.16	MS, RI
9.80	**1286**	**Octanal**	124-13-0	-	0.08	MS, RI, S
10.27	**1305**	**4,8-Dimethyl-1,3,7-nonatriene**	51911-82-1	1.66	1.54	MS, RI
10.53	**1315**	***(Z)*-3-Hexenol acetate**	3681-71-8	-	0.08	MS, RI
10.65	**1319**	***(Z)*-2-Heptenal**	57266-86-1	-	0.10	MS, RI
11.03	**1335**	**Sulcatone**	110-93-0	-	0.02	MS, RI
11.41	**1350**	**1-Hexanol**	111-27-3	-	0.08	MS, RI, S
11.68	**1360**	**Rose oxide**	16409-43-1	-	0.01	MS, RI
12.19	**1381**	***(Z)*-3-Hexenol**	928-96-1	-	0.24	MS, RI
12.31	**1385**	**2-Nonanone**	821-55-6	-	0.01	MS, RI
12.44	**1390**	**Nonanal**	124-19-6	-	0.16	MS, RI
13.14	**1418**	**1,3,8-ρ-Menthatriene**	18368-95-1	-	0.02	MS, RI
13.30	**1425**	***(E)*-2-Octenal**	2548-87-0	-	0.10	MS, RI
13.50	**1433**	**Dehydro-ρ-cymene**	1195-32-0	-	0.23	MS, RI
13.78	**1444**	**Cosmene**	460-01-5	0.48	0.04	MS, RI
13.90	**1449**	**1-Octen-3-ol**	3391-86-4	-	0.03	MS, RI

13.96	**1451**	**α-Cubebene**	17699-14-8	0.75	0.19	MS, RI
14.24	**1462**	**δ-Elemene**	20307-84-0	-	0.87	MS, RI
14.52	**1473**	**Octyl acetate**	112-14-1	6.31	0.30	MS, RI
14.86	**1487**	**2-Ethyl-1-hexanol**	104-76-7	-	0.23	MS, RI
15.07	**1495**	**Decanal**	112-31-2	0.19	0.11	MS, RI
15.29	**1504**	**Camphor**	464-49-3	-	0.03	MS, RI
15.36	**1508**	**α-Copaene**	3856-25-5	0.24	0.19	MS, RI
15.39	**1509**	**β-Cubebene**	13744-15-5	2.41	-	MS, RI
15.51	**1514**	**2-Bornene**	464-17-5	-	0.07	MS, RI
15.61	**1518**	**Benzaldehyde**	100-52-7	-	0.05	MS, RI
16.31	**1547**	**Linalool**	78-70-6	-	0.34	MS, RI, S
16.51	**1555**	**1-Octanol**	111-87-5	-	0.98	MS, RI
16.95	**1573**	**2,3-Butanediol**	513-85-9	-	0.01	MS, RI
17.01	**1576**	**Nonanol acetate**	143-13-5	0.89	0.12	MS, RI
17.08	**1579**	**Fenchol**	1632-73-1	-	0.06	MS, RI
17.20	**1584**	**β-Elemene**	515-13-9	-	0.23	MS, RI
17.27	**1587**	**β-Caryophyllene**	87-44-5	-	0.07	MS, RI
17.42	**1593**	**Sibirene**	14029-18-6	2.09	-	MS, RI
17.50	**1596**	**Terpinen-4-ol**	562-74-3	-	0.35	MS, RI, S
17.78	**1615**	**β-Copaene**	18252-44-3	0.51	0.07	MS, RI
18.34	**1662**	**γ-Elemene**	3242-08-8	2.27	0.50	MS, RI
18.47	**1672**	**α-Elemene**	5951-67-7	-	0.10	MS, RI
18.67	**1688**	**Germacrene D**	23986-74-5	11.21	-	MS, RI
18.87	**1702**	**Valencene**	4630-07-3	-	0.58	MS, RI
18.92	**1703**	**1-Nonanol**	143-08-8	-	0.32	MS, RI
18.97	**1705**	**α-Caryophyllene**	6753-98-6	-	0.13	MS, RI

19.03	**1707**	**Bicyclosesquiphellandrene**	54324-03-7	-	0.08	MS, RI
19.10	**1709**	**β-Farnesene**	77129-48-7	6.26	0.79	MS, RI, S
19.42	**1718**	**Decyl acetate**	112-17-4	-	0.49	MS, RI
19.46	**1720**	**γ-Muurolene**	30021-74-0	3.29	0.64	MS, RI
19.58	**1723**	**β-Cadinene**	523-47-7	3.83	0.79	MS, RI
19.72	**1728**	**α-Terpineol**	98-55-5	-	0.69	MS, RI, S
20.28	**1745**	**α-Muurolene**	10208-80-7	-	0.28	MS, RI
20.45	**1750**	**Carvone**	99-49-0	-	0.11	MS, RI, S
20.60	**1754**	***(Z)*-Carvyl acetate**	1205-42-1	-	0.10	MS, RI
20.96	**1765**	**α-Farnesene**	502-61-4	-	0.09	MS, RI, S
21.03	**1767**	**δ-Cadinene**	483-76-1	-	1.73	MS, RI
21.11	**1770**	**Geranyl acetate**	105-87-3	-	0.11	MS, RI, S
21.23	**1773**	**1-Decanol**	112-30-1	-	0.37	MS, RI, S
21.32	**1776**	**Citronellol**	106-22-9	-	0.12	MS, RI, S
21.44	**1779**	**Selina-3,7(11)-dien**	6813-21-4	0.53	0.17	MS, RI
21.53	**1782**	**Cubenene**	29837-12-5	-	0.09	MS, RI
21.76	**1789**	**α-Cadinene**	24406-05-1	0.58	0.18	MS, RI
22.04	**1798**	**Nerol**	106-25-2	-	0.04	MS, RI
22.49	**1818**	**Germacrene D**	15423-57-1	3.34	0.32	MS, RI
22.61	**1823**	***(E)*-Calamenene**	73209-42-4	0.11	0.04	MS, RI
22.65	**1825**	***(Z)*-Calamenene**	483-77-2	0.02	0.03	MS, RI
22.76	**1830**	**Isopiperitenone**	529-01-1	-	0.06	MS, RI
22.83	**1834**	**Isopropyl laurate**	10233-13-3	-	0.06	MS, RI
23.00	**1842**	**α-Isomethyl ionone**	127-51-5	-	0.01	MS, RI

23.06	**1844**	**Geraniol**	106-24-1	-	0.04	MS, RI, S
23.21	**1852**	***(E)*-Geranylacetone**	3796-70-1	-	0.04	MS, RI
23.33	**1857**	**Hexanoic acid**	142-62-1	-	0.12	MS, RI, S
24.82	**1929**	**ρ-Mentha-1-en-9-ol**	18479-68-0	-	0.01	MS, RI
25.41	**1958**	**Ethylhexanoic acid**	149-57-5	-	0.01	MS, RI
25.55	**1964**	**1-Dodecanol**	112-53-8	-	0.08	MS, RI
26.22	**1997**	**Perilla alcohol**	536-59-4	-	0.00	MS, RI, S
27.01	**2039**	***(E)*-Nerolidol**	40716-66-3	0.12	0.02	MS, RI, S
27.16	**2047**	**Junenol**	472-07-1	0.04	0.23	MS, RI
27.62	**2068**	**Viridiflorol**	552-02-3	0.14	0.22	MS, RI
28.05	**2095**	**10-epi-γ-Eudesmol**	15051-81-7	0.01	0.02	MS, RI
28.29	**2108**	**Cedrol**	77-53-2	-	0.02	MS, RI, S
28.73	**2133**	**Neointermedeol**	5945-72-2	0.04	0.00	MS, RI
29.31	**2165**	**γ-Eudesmol**	1209-71-8	-	0.02	MS, RI
29.37	**2169**	**Cedrelanol**	5937-11-1	-	0.02	MS, RI
29.66	**2185**	**δ-Cadinol**	19435-97-3	-	0.12	MS, RI
29.87	**2197**	**τ-Muurolol**	19912-62-0	0.03	0.03	MS, RI
30.11	**2210**	**Hexyl salicylate**	6259-76-3	-	0.00	MS, RI
30.25	**2219**	**α-Eudesmol**	473-16-5	0.02	0.02	MS, RI
30.40	**2227**	**β-Eudesmol**	473-15-4	-	0.06	MS, RI
30.46	**2231**	**α-Cadinol**	481-34-5	0.15	0.05	MS, RI
30.90	**2256**	**Isospathulenol**	88395-46-4	-	0.01	MS, RI

The identified volatiles in the zest are shown in Figure 3 and Table 4. A total of 83 volatiles were identified in the zest which consisted of 7 esters, 8 aldehydes, 53 terpenes, 12 alcohols, 2 ketones, and 1 acid. Major volatiles identified in the zest included limonene (5.15 – 13.38%), germacrene D (0.03 – 9.31%), geranyl acetate (1.04 – 6.60%), α-pinene (4.60 – 6.06%), β-myrcene (2.44 – 4.54%), linalool (2.09 – 4.36%), decanal (0.86 – 4.14%), and 1-octanol (1.30 – 2.55%), accounting for 29.30 – 39.15% of the total volatiles.

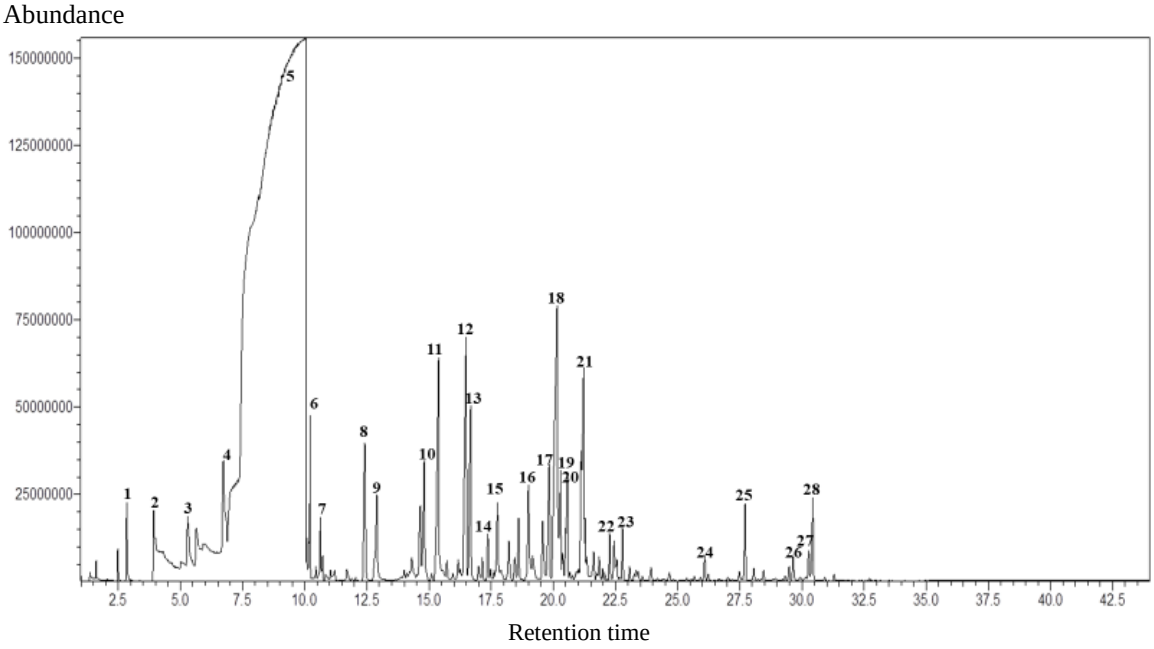

Figure 3. SPME-GC-MS extraction of calamondin zest.

Key: (1) ethanol (2) α-pinene (3) β-pinene (4) β-myrcene (5) limonene (6) *(E)*-β-ocimene (7) terpinolene (8) (*Z*)-3-hexenol (9) nonanal (10) octyl acetate (11) decanal (12) linalool (13) 1-octanol (14) β-elemene (15) undecanal (16) 1-nonanol (17) α-terpineol (18) germacrene D (19) β-selinene (20) bicyclogermacrene (21) geranyl acetate (22) (*E,E*)-2,4-decadienal (23) isopiperitenone (24) perilla alcohol (25) β-elemol (26) γ-eudesmol (27) α-eudesmol (28) β-eudesmol.

Table 4

Volatile compounds with SPME from calamondin zest using polar column ZB Wax column (30 m x 0.25 mm x 0.25 µm).

RT (min)	LRI	Compound Name	CAS #	April Area %	August Area %	ID Criteria
1.61	**721**	**Acetaldehyde**	75-07-0	-	0.12	MS, RI
2.06	**777**	**Methyl acetate**	79-20-9	-	0.03	MS, RI
2.43	**869**	**Ethyl acetate**	141-78-6	-	0.00	MS, RI
2.48	**886**	**Methyl alcohol**	67-56-1	0.00	0.23	MS, RI
2.77	**921**	**Isopropyl alcohol**	67-63-0	-	0.00	MS, RI
2.84	**927**	**Ethanol**	64-17-5	0.03	0.75	MS, RI
3.36	**971**	**3-Pentanone**	96-22-0	-	0.01	MS, RI
4.46	**1044**	**α-Pinene**	80-56-8	6.06	4.60	MS, RI
6.09	**1130**	**β-Pinene**	127-91-3	0.88	0.83	MS, RI
6.23	**1136**	**Pseudolimonene**	499-97-8	-	0.05	MS, RI
6.33	**1141**	**Sabinene**	3387-41-5	-	0.12	MS, RI
6.97	**1169**	**β-Myrcene**	123-35-3	4.54	2.44	MS, RI
8.18	**1221**	**Limonene**	138-86-3	13.38	5.15	MS, RI, S
10.09	**1298**	***(E)*-β-Ocimene**	3779-61-1	0.03	0.06	MS, RI
10.18	**1301**	**γ-Terpinene**	99-85-4	0.12	0.23	MS, RI
10.38	**1309**	**o-Cymene**	527-84-4	-	0.10	MS, RI
10.54	**1315**	**Isoterpinolene**	586-63-0	0.01	0.03	MS, RI
10.63	**1319**	**Terpinolene**	586-62-9	0.14	0.43	MS, RI
10.72	**1322**	**Octanal**	124-13-0	0.29	0.20	MS, RI, S
10.84	**1327**	***(Z)*-2-Pentenol**	1576-95-0	-	0.09	MS, RI
11.05	**1335**	***(E)*-4,8-Dimethylnona-1,3,7-triene**	19945-61-0	0.01	0.10	MS, RI
11.21	**1342**	***(Z)*-3-Hexenyl acetate**	3681-71-8	0.05	0.17	MS, RI
11.69	**1361**	**Hexanol**	111-27-3	0.03	0.19	MS, RI, S
11.89	**1369**	***(E)*-3-Hexenol**	928-97-2	0.00	0.04	MS, RI
12.42	**1390**	***(Z)*-3-Hexenol**	928-96-1	0.37	2.10	MS, RI

12.90	**1409**	**Nonanal**	124-19-6	0.34	1.80	MS, RI
13.48	**1432**	**Perillene**	539-52-6	-	0.01	MS, RI
13.87	**1447**	**Dehydro-ρ-cymene**	1195-32-0	0.02	0.03	MS, RI
14.00	**1453**	***(Z)*-Limonene oxide**	13837-75-7	-	0.09	MS, RI
14.10	**1457**	**Cosmene**	460-01-5	0.02	0.14	MS, RI
14.30	**1465**	***(E)*-Limonene oxide**	4959-35-7	0.01	0.58	MS, RI
14.65	**1479**	**δ-Elemene**	20307-84-0	0.46	1.75	MS, RI
14.81	**1485**	**Octyl acetate**	112-14-1	0.76	1.90	MS, RI
15.11	**1497**	**α-Copaene**	3856-25-5	0.17	0.14	MS, RI
15.39	**1508**	**Decanal**	112-31-2	0.86	4.14	MS, RI, S
15.65	**1519**	**β-Bourbonene**	5208-59-3	-	0.61	MS, RI
16.18	**1541**	**β-Cubebene**	13744-15-5	1.96	0.69	MS, RI
16.49	**1554**	**Linalool**	78-70-6	2.09	4.36	MS, RI, S
16.68	**1562**	**1-Octanol**	111-87-5	1.30	2.55	MS, RI
17.15	**1582**	**Nonanol acetate**	143-13-5	0.10	0.31	MS, RI
17.37	**1591**	**β-Elemene**	515-13-9	0.26	0.84	MS, RI
17.48	**1595**	**β-Caryophyllene**	87-44-5	0.03	0.12	MS, RI
17.62	**1602**	**Terpinen-4-ol**	562-74-3	0.02	0.08	MS, RI, S
17.76	**1614**	**Undecanal**	112-44-7	0.18	1.45	MS, RI
18.21	**1651**	***(E)*-2,8-ρ-Mentha-dien-1-ol**	7212-40-0	-	0.62	MS, RI
18.45	**1671**	**γ-Muurolene**	30021-74-0	0.13	0.49	MS, RI
18.60	**1683**	***(E)*-2-Decenal**	3913-81-3	0.09	0.76	MS, RI
19.00	**1706**	**1-Nonanol**	143-08-8	0.56	1.82	MS, RI
19.42	**1719**	**Citral**	5392-40-5	-	0.03	MS, RI, S
19.61	**1724**	**Decyl acetate**	112-17-4	0.25	1.30	MS, RI
19.84	**1731**	**α-Terpineol**	98-55-5	0.46	2.10	MS, RI, S

20.15	**1741**	**Germacrene D**	23986-74-5	0.03	9.31	MS, RI
20.34	**1746**	**β-Selinene**	17066-67-0	0.44	2.31	MS, RI
20.60	**1754**	**Bicylogermacrene**	24703-35-3	0.11	1.95	MS, RI
21.25	**1774**	**Geranyl acetate**	105-87-3	1.04	6.60	MS, RI, S
21.63	**1785**	**Perillaldehyde**	2111-75-3	0.07	0.54	MS, RI
21.85	**1792**	**γ-Cadinene**	39029-41-9	-	0.49	MS, RI
21.98	**1796**	***(Z)*-4-Decenol**	57074-37-0	0.01	0.22	MS, RI
22.27	**1807**	**(*E,E*)-2,4-Decadienal**	25152-84-5	0.06	0.54	MS, RI
22.45	**1816**	***(E)*-2-Undecen-1-ol**	75039-84-8	-	0.59	MS, RI
22.51	**1819**	**Germacrene B**	15423-57-1	-	0.52	MS, RI
22.79	**1832**	**Isopiperitenone**	529-01-1	-	0.51	MS, RI
23.07	**1845**	**Geraniol**	106-24-1	0.11	0.17	MS, RI, S
23.33	**1857**	***(E)*-2-Dodecenal**	20407-84-5	-	0.39	MS, RI
23.94	**1886**	***(E,Z)*-2,6-Dodecadienal**	21662-13-5	-	0.28	MS, RI
24.20	**1898**	**Perillyl acetate**	15111-96-3	0.02	0.04	MS, RI
24.41	**1909**	**α-Calacorene**	21391-99-1	0.01	0.08	MS, RI
24.84	**1930**	**ρ-Mentha-1-en-9-ol**	18479-68-0	0.00	0.04	MS, RI
25.56	**1965**	**1-Dodecanol**	112-53-8	0.00	0.03	MS, RI
26.23	**1998**	**Perillyl alcohol**	536-59-4	0.04	0.11	MS, RI, S
27.04	**2041**	***(E)*-Nerolidol**	40716-66-3	0.00	0.08	MS, RI, S
27.72	**2078**	**Elemol**	639-99-6	0.08	1.01	MS, RI
27.88	**2086**	**Guaiol**	489-86-1	0.00	0.02	MS, RI

29.34	**2167**	**γ-Eudesmol**	1209-71-8	0.02	0.11	MS, RI
30.28	**2220**	**α-Eudesmol**	473-16-5	0.05	0.53	MS, RI
30.44	**2230**	**β-Eudesmol**	473-15-4	0.11	1.10	MS, RI
30.92	**2258**	**Isospathulenol**	88395-46-4	0.00	0.06	MS, RI
31.30	**2279**	**Limonene-1,2-diol**	1946-00-5	0.01	0.07	MS, RI
31.99	**2321**	**8-Hydroxylinalool**	64142-78-5	-	0.00	MS, RI
34.20	**2456**	**Indole**	120-72-9	0.00	0.00	MS, RI
34.71	**2488**	**Benzophenone**	119-61-9	-	0.00	MS, RI
34.97	**2504**	**Thunbergol**	25269-17-4	-	0.00	MS, RI
42.21	**2886**	**Hexadecanoic acid**	57-10-3	-	0.00	MS, RI

Although the number of volatiles identified in the zest was less than that in the juice, the total peak intensity in the zest was 1.2×10^{11}, while in the juice was 3.8×10^{10}. Volatile intensity in zest was more than three times over that in juice. Overall, major compounds identified in this study are limonene, decanal, linalool, and β-myrcene. SPME analysis of the calamondin juice has been reported in literature with limonene and myrcene as the major compounds identified (Cheong et al., 2012b; Takeuchi et al., 2005; Yamamoto et al., 2012; Yo et al., 2004).

Analysis of the juice and peel from harvests in April and August varied in compounds and their % peak area. Comparison of the juice from both harvests shared the following 34 compounds: β-pinene, β-myrcene, limonene, β-phellandrene, *(E)*-β-ocimene, γ-terpinene, *(Z)*-β-ocimene, ρ-cymene, α-terpinolene, 4,8-dimethyl-1,3,7-nonatriene, cosmene, α-cubebene, octyl acetate, decanal, α-copaene, nonanol acetate, β-copaene, γ-

elemene, β-farnesene, γ-muurolene, β-cadinene, selina-3,7(11)-dien, α-cadinene, germacrene D, *(E)*-calamenene, *(Z)*-calamenene, *(E)*-nerolidol, junenol, viridiflorol, 10-epi-γ-eudesmol, neointermedeol, τ-muurolol, α-eudesmol, and α-cadinol. The April harvest of the 34 compounds accounted for 72.41% peak area and total peak intensity of 6.3×10^{10} compared to the August harvest at 73.39% peak area with total peak intensity of 1.3×10^{10}. Significant differences in concentrations were in compounds β-pinene in the April harvest at 4.95% and 0.43% in the August harvest, limonene in the April harvest at 30.81% and 62.11% in the August harvest, octyl acetate in the April harvest at 6.31% and 0.30% in the August harvest, and β-farnesene in the April harvest at 6.26% and 0.79% in the August harvest. Germacrene D was identified in the April harvest at 11.21% whereas the compound was not detected in the August harvest. Higher concentrations of β-myrcene, γ-terpinene, *(Z)*-β-ocimene, 4,8-dimethyl-1,3,7-nonatriene, cosmene, α-cubebene, decanal, α-copaene, nonanol acetate, β-copaene, γ-elemene, γ-muurolene, β-cadinene, Selina-3,7(11)-dien, α-cadinene, germacrene D, *(E)*-calamene, *(E)*-nerolidol, neointermedeol, and α-cadinol were found in the April harvest than August harvest. β-phellandrene, *(E)*-β-ocimene, ρ-cymene, α-terpinolene, *(Z)*-calamene, junenol, viridiflorol, and 10-epi-γ-eudesmol showed a decrease of their concentrations in April harvest compared to August harvest while the concentrations of τ-muurolol and α-eudesmol shared the same concentration.

Comparison of the zest from both harvests shared the following 59 compounds: methyl alcohol, ethanol, α-pinene, β-pinene, β-myrcene, limonene, *(E)*-β-ocimene, γ-

terpinene, isoterpinolene, terpinolene, octanal, *(E)*-4,8-dimethylnona-1,3,7-triene, *(Z)*-3-hexenyl acetate, hexanol, *(E)*-3-hexenol, *(Z)*-3-hexenol, nonanal, ρ-α-dimethyl styrene, cosmene, *(E)*-limonene oxide, δ-elemene, octyl acetate, α-copaene, decanal, β-cubebene, linalool, 1-octanol, nonanol acetate, β-elemene, β-caryophyllene, terpinen-4-ol, undecanal, γ-muurolene, *(E)*-2-decenal, 1-nonanol, decyl acetate, α-terpineol, germacrene D, β-selinene, bicylogermacrene, geranyl acetate, perillaldehyde, *(Z)*-4-decenol, (*E,E*)-2,4-decadienal, geraniol, perillyl acetate, α-calacorene, ρ-mentha-1-en-9-ol, 1-dodecanol, perillyl alcohol, *(E)*-nerolidol, elemol, guaiol, γ-eudesmol, α-eudesmol, β-eudesmol, isospathulenol, limonene-1,2-diol, and indole. The April harvest of the 59 compounds accounted for 38.22% peak area of total volatiles and total peak intensity of 1.5×10^{11} compared to the August harvest at 69.82% peak area with total peak intensity of 9.4×10^{10}. Significant difference in quantities were found in limonene in the April harvest at 13.38% and 5.15% in the August harvest, decanal in the April harvest at 0.86% and 4.14% in the August harvest, germacrene D in the April harvest at 0.03% and 9.31% in the August harvest, and geranyl acetate in the April harvest at 1.04% and 6.60% in the August harvest. Higher concentrations of α-pinene, β-pinene, β-myrcene, octanal, α-copaene, and β-cubebene were found in the April harvest than August harvest. Methyl alcohol, ethanol, *(E)*-β-ocimene, γ-terpinene, isoterpinolene, terpinolene, *(E)*-4,8-dimethylnona-1,3,7-triene, *(Z)*-3-hexenyl acetate, hexanol, *(E)*-3-hexenol, *(Z)*-3-hexenol, nonanal, ρ-α-dimethyl styrene, cosmene, *(E)*-limonene oxide, δ-elemene, octyl acetate, linalool, 1-octanol, nonanol acetate, β-elemene, β-caryophyllene, terpinen-4-ol, undecanal, γ-

muurolene, *(E)*-2-decenal, 1-nonanol, decyl acetate, α-terpineol, β-selinene, bicylogermacrene, perillaldehyde, *(Z)*-4-decenol, (*E*,*E*)-2,4-decadienal, geraniol, perillyl acetate, α-calacorene, ρ-mentha-1-en-9-ol, 1-dodecanol, perillyl alcohol, *(E)*-nerolidol, elemol, guaiol, γ-eudesmol, α-eudesmol, β-eudesmol, isospathulenol, and limonene-1,2-diol were identified at lower levels in April harvest compared to August harvest while the concentration of indole shared the same concentration.

SPE Solvent Elution Comparison

SPE of the calamondin juice using elution solvents 100% methanol and 95:5, 90:10, 85:15, and 75:25 dichloromethane:methanol ratios were compared and chromatographs for the different solvents are indicated in Figure 4. Chromatograph displaying 100% methanol had 10 significant peaks, 95:5 dichloromethane:methanol had 28 significant peaks, 90:10 dichloromethane:methanol had 13 significant peaks, 85:15 dichloromethane:methanol had 19 significant peaks, and 75:25 dichloromethane:methanol had 13 significant peaks. Volatile identification showed major volatiles of limonene, linalool, and alpha-terpineol were present in various quantities in all of the extractions, with the 95:5 dichloromethane:methanol elution producing the highest peak areas of each major volatile.

Analysis also revealed compounds known as artifacts, which are byproducts of thermal degradation of organic compounds such as sugars and pigments in extracts usually formed due to the high temperature at the GC injection port. Known artifacts included furfural, 5-methyl-2-furancarboxaldehyde, 2H-pyran-2,6(3H)-dione, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 2,3-dihydro-benzofuran, 5-hydroxymethylfurfural, and

guaiacol. Artifacts present in the extractions indicate that the method of extraction produced an unrefined sample and may not be ideal for GC-MS analysis as these compounds although detected, are not actually compounds in the sample. Artifacts were identified in 4 out of the 5 extractions, with the exception being the 95:5 dichloromethane:methanol elution.

Chromatographic results also showed that the 95:5 dichloromethane:methanol solvent elution has the most peaks compared to the other solvents, indicating the elution extracted a wide range of volatiles qualitatively and quantitatively. In addition, no artifacts were present in the 95:5 dichloromethane:methanol solvent elution. Therefore, this combination of solvent was selected as the next step.

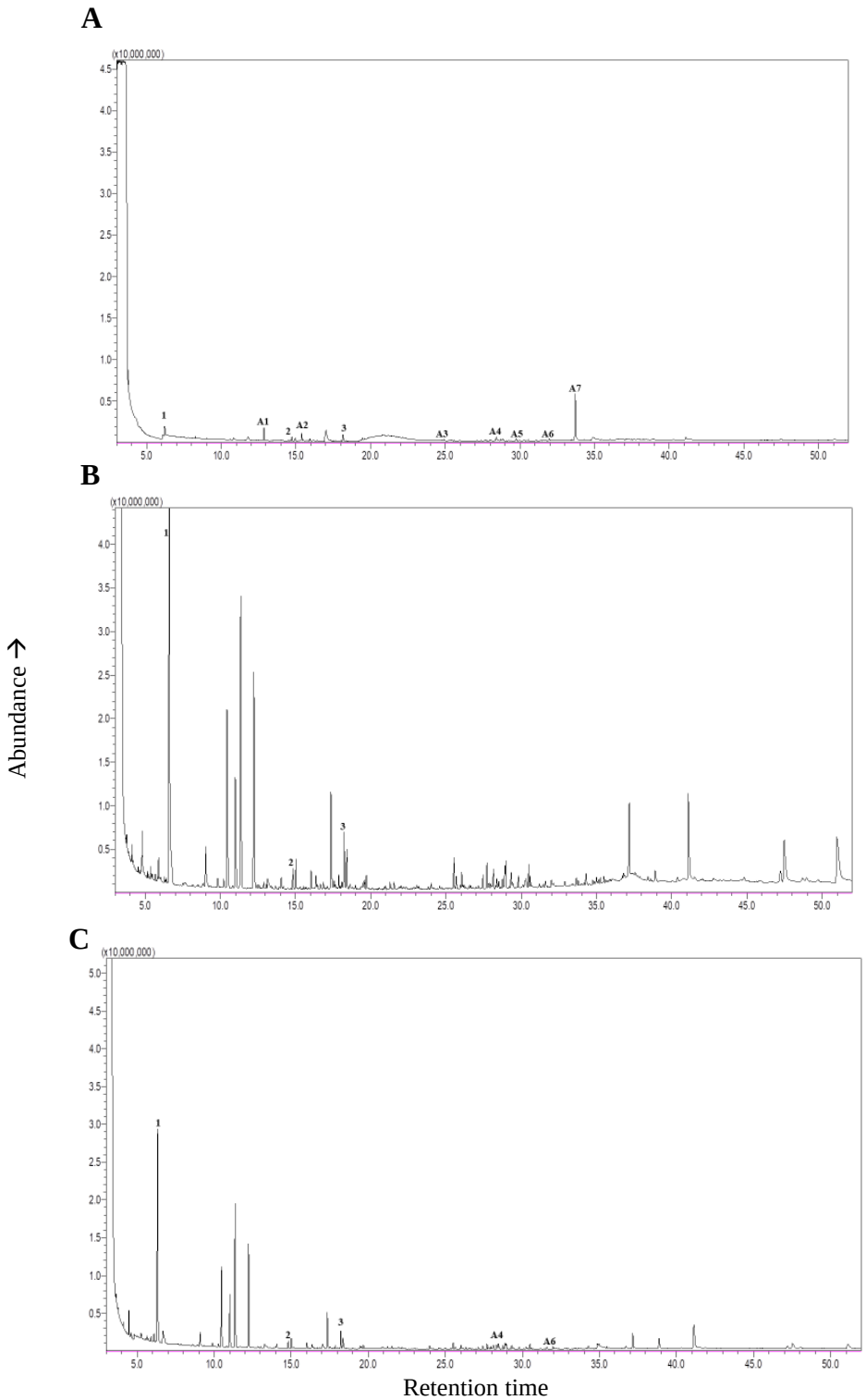

Figure 4. LiChrolut-EN SPE-GC-MS with (A) 100% methanol (B) 95:5 dichloromethane:methanol (C) 90:10 dichloromethane:methanol elution of calamondin juice.

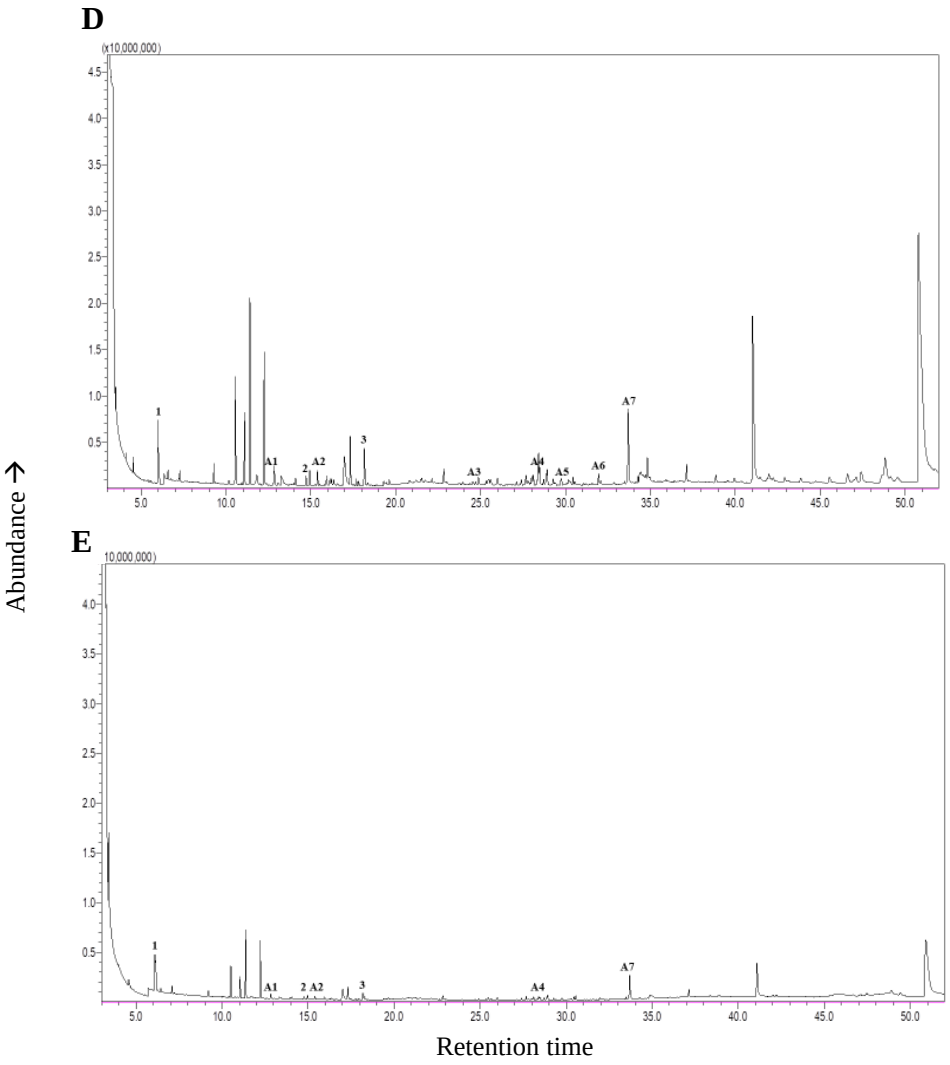

Figure 4 continued. LiChrolut-EN SPE-GC-MS with (A) 100% methanol (B) 95:5 dichloromethane:methanol (C) 90:10 dichloromethane:methanol (D) 85:15 dichloromethane:methanol (E) 75:25 dichloromethane:methanol elution of calamondin juice.
Key: (1) limonene (2) linalool (3) α-terpineol
Artifacts – (A1) furfural (A2) 5-methyl-2-furancarboxaldehyde (A3) 2H-pyran-2,6(3H)-dione (A4) guaiacol (A5) 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (A6) 2,3-dihydro-benzofuran (A7) 5-hydroxymethylfurfural.

95:5 Dichloromethane:Methanol Solvent Elution – 30 mL vs 1 mL

With analysis indicating the 95:5 dichloromethane:methanol 30 mL solvent elution produced optimum extraction results, 1 mL elution with the solvent was compared. These different elution volumes were compared to observe volatile extraction efficacy. Volume 1 mL of the solvent was used to eluate the samples followed by GC-MS analysis, therefore skipping distillation procedure that would be used with 30 mL elution.

Figure 5 shows that the 30 mL solvent elution extracted approximately 20 peaks, 8 more significant peaks compared to the 1 mL solvent elution which displays 12. Total peak intensity for 30 mL elution is higher at 9.9×10^9 and the 1 mL elution is 3.8×10^9. Area percentage of compounds found in both extractions indicated that the 30 mL elution extracted a higher concentration of compounds such as: 33.81% more limonene, 13.59% more linalool, 70.62% more 1-nonanol, 20.64% more α-terpineol, 92.42% more 1-decanol, 339.87% more neointermedeol, 22.82% more limonene-1,2-diol, 107.32% more 8-hydroxylinalool, and 784.77% more hexadecanoic acid. According to the following results for both calamondin juice and peel, 30 mL of 95:5 dichloromethane:methanol was used for volatile extraction method.

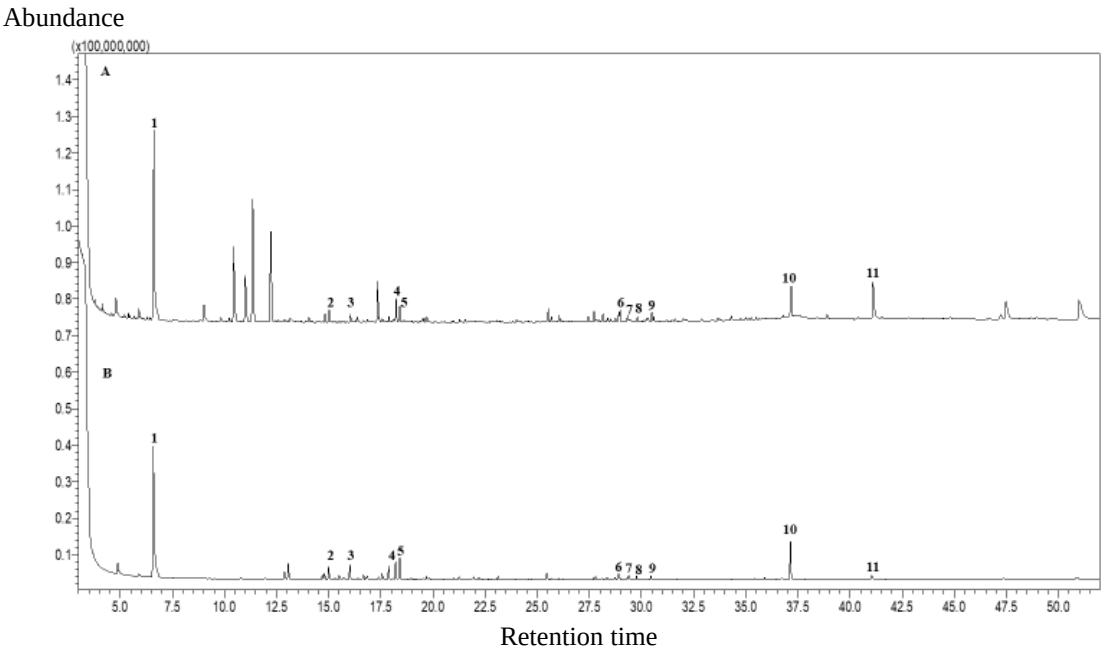

Figure 5. LiChrolut-EN SPE-GC-MS with (A) 30 mL of 95:5 dichloromethane:methanol (B) 1 mL of 95:5 dichloromethane:methanol elution of calamondin juice.

Key: (1) limonene (2) linalool (3) terpinen-4-ol (4) α-terpineol (5) β-cubebene (6) β-eudesmol (7) neointermedeol (8) limonene-1,2-diol (9) 8-hydroxylinalool (10) cryptomeridiol (11) hexadecanoic acid.

Volatile Composition in Calamondin Juice and Peel using SPE-GC-MS

Using SPE with 30 mL elution of 95:5 dichloromethane:methanol solution on the juice, 75 volatiles were identified, as shown in Figure 6 and Table 5. Compounds comprise of 37 terpenes, 10 alcohols, 4 aldehydes, 8 acids, 2 esters, 2 ketones, 1 furan, 7 phenols, 1 N-containing compound, 1 O-containing compound, and 2 norisoprenoids. Major volatiles detected in the juice are limonene (14.51 – 14.59%), hexadecanoic acid (3.19 – 10.88%), 4-hydroxy-benzeneethanol (0.09 – 7.98%), cryptomeridiol (4.95 – 5.76%), stearic acid

(3.38 – 3.82%), α-terpineol (2.29 – 3.76%), *(Z)*-8-hydroxylinalool (0.45 – 3.58%), α-cadinol (1.23 – 3.16%), limonen-1,2-diol (0.41 – 2.85%), linoleic acid (1.36 – 2.73%), and *(Z)*-3-hexenol (0.17 – 1.36%), accounting for 33.96 – 58.54% of total volatiles. Limonene is characterized by a cool, fresh and minty aroma and *(Z)*-3-hexenol is like the aroma of fresh cut grass.

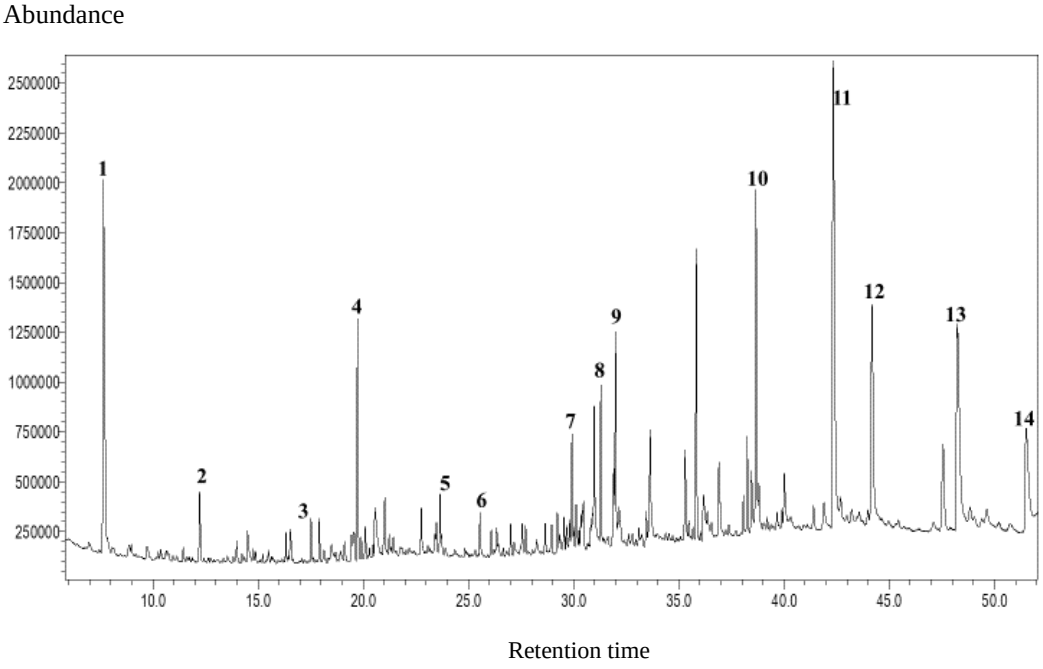

Figure 6. LiChrolut-EN SPE-GC-MS with 95:5 dichloromethane:methanol elution of calamondin juice.

Key: (1) limonene (2) (*Z*)-3-hexenol (3) terpinen-4-ol (4) α-terpineol (5) benzyl alcohol (6) dodecanol (7) 2-methoxy-4-vinylphenol (8) limonen-1,2-diol (9) (*Z*)-8-hydroxylinalool (10) cryptomeridiol (11) hexadecanoic acid (12) 4-hydroxy-benzeneethanol (13) stearic acid (14) linoleic acid.

Table 5

Volatile compounds in SPE with 95:5 dichloromethane:methanol elution from calamondin juice using polar column ZB Wax column (30 m x 0.25 mm x 0.25 µm).

RT (min)	LRI	Compound Name	CAS #	April Area %	August Area %	ID Criteria
6.96	**1137**	**Myrcene**	123-35-3	0.16	0.14	MS, RI
7.65	**1167**	**Limonene**	138-86-3	14.51	14.59	MS, RI
9.73	**1254**	**Acetoin**	513-86-0	-	0.37	MS, RI
10.37	**1281**	**4,8-Dimethyl-1,3,7-nonatriene**	51911-82-1	0.07	0.22	MS, RI
10.63	**1291**	**Prenol**	556-82-1	0.03	0.16	MS, RI
11.45	**1324**	**Hexanol**	111-27-3	-	0.25	MS, RI, S
12.22	**1355**	**(*Z*)-3-Hexenol**	928-96-1	0.17	1.36	MS, RI
12.42	**1363**	**(*E*)-ρ-2,8-Menthadien-1-ol**	7212-40-0	-	0.04	MS, RI
14.50	**1449**	**Acetic acid**	79-09-4	-	0.75	MS, RI
14.77	**1460**	**Isomenthone**	491-07-6	-	0.26	MS, RI
14.88	**1465**	**2-Ethyl-1-hexanol**	104-76-7	0.07	0.16	MS, RI
15.64	**1498**	**Benzaldehyde**	100-52-7	0.13	0.11	MS, RI
16.32	**1527**	**Linalool**	78-70-6	0.55	0.41	MS, RI, S
16.52	**1536**	**1-Octanol**	111-87-5	0.89	0.80	MS, RI
17.10	**1561**	**Fenchol**	1632-73-1	0.13	0.04	MS, RI
17.52	**1579**	**Terpinen-4-ol**	562-74-3	1.08	0.71	MS, RI, S
18.14	**1606**	**(*Z*)-ρ-Mentha-2,8-dien-1-ol**	22771-44-4	-	0.28	MS, RI
18.35	**1616**	**γ-Elemene**	3242-08-8	-	0.09	MS, RI
18.42	**1619**	**Menthol**	89-78-1	-	0.05	MS, RI, S
18.50	**1623**	**Benzeneacetaldehyde**	122-78-1	-	0.23	MS, RI
18.93	**1642**	**1-Nonanol**	143-08-8	0.19	0.18	MS, RI
18.99	**1645**	**2-Furanmethanol**	98-00-0	-	0.09	MS, RI
19.12	**1651**	**β-Caryophyllene**	87-44-5	-	0.44	MS, RI, S

19.42	**1665**	**Decyl acetate**	112-17-4	1.18	0.34	MS, RI
19.47	**1667**	**γ-Muurolene**	30021-74-0	0.14	0.36	MS, RI
19.53	**1670**	**Isovaleric acid**	503-74-2	-	0.42	MS, RI
19.73	**1678**	**α-Terpineol**	98-55-5	2.29	3.76	MS, RI, S
20.29	**1704**	**α-Amorphene**	483-75-0	0.17	0.13	MS, RI
20.47	**1713**	**Carvone**	99-49-0	0.07	0.18	MS, RI, S
20.97	**1736**	**Isopiperitenol**	491-05-4	0.03	0.25	MS, RI
21.03	**1739**	**α-Muurolene**	10208-80-7	0.12	0.97	MS, RI
21.24	**1749**	**Decanol**	112-30-1	0.29	0.37	MS, RI, S
21.32	**1753**	**Citronellol**	106-22-9	-	0.09	MS, RI, S
21.43	**1758**	**Selina-4(15),7(11)-diene**	515-17-3	-	0.26	MS, RI
22.05	**1787**	**Nerol**	106-25-2	-	0.06	MS, RI
22.20	**1794**	**3,4-Dimethyl-benzaldehyde**	5973-71-7	-	0.07	MS, RI
22.31	**1800**	**3-Methyl-2-butenoic acid**	541-47-9	-	0.05	MS, RI
22.76	**1822**	**Isopiperitenone**	529-01-1	0.28	0.76	MS, RI
22.84	**1826**	**Isopropyl dodecanoate**	10233-13-3	-	0.08	MS, RI
23.07	**1837**	**Geraniol**	106-24-1	0.13	0.11	MS, RI, S
23.41	**1854**	**Hexanoic acid**	142-62-1	0.24	0.40	MS, RI, S
23.65	**1866**	**Benzyl alcohol**	100-51-6	-	0.97	MS, RI
23.90	**1878**	**(*Z*)-ρ-Mentha-1(7),8-dien-2-ol**	29548-13-8	-	0.11	MS, RI
24.36	**1901**	**Phenylethyl alcohol**	60-12-8	-	0.09	MS, RI
24.83	**1925**	**ρ-Menth-1-en-9-ol**	18479-68-0	0.12	0.12	MS, RI
25.55	**1962**	**Dodecanol**	112-53-8	0.28	0.78	MS, RI
26.22	**1997**	**Perilla alcohol**	536-59-4	-	0.07	MS, RI, S
26.65	**2020**	**2-Pyrrolidinone**	616-45-5	0.05	0.14	MS, RI
27.56	**2069**	**Viridiflorol**	552-02-3	0.70	0.56	MS, RI

28.05	**2095**	**γ-Eudesmol**	1209-71-8	-	0.05	MS, RI
29.47	**2174**	**Hinesol**	23811-08-7	-	0.11	MS, RI
29.66	**2185**	**α-Cadinol**	481-34-5	3.16	1.23	MS, RI
29.92	**2199**	**2-Methoxy-4-vinylphenol**	7786-61-0	-	2.06	MS, RI
30.83	**2252**	**Intermedeol**	6168-59-8	0.46	0.41	MS, RI
31.29	**2279**	**Limonen-1,2-diol**	1946-00-5	0.41	2.85	MS, RI
31.63	**2299**	**Decanoic acid**	334-48-5	-	0.17	MS, RI
31.82	**2310**	**4-Methyl-5-thiazoleethanol**	137-00-8	-	0.31	MS, RI
31.99	**2320**	**(*Z*)-8-Hydroxylinalool**	103619-06-3	0.45	3.58	MS, RI
32.61	**2358**	**(*E*)-Isoeugenol**	5932-68-3	0.03	0.21	MS, RI
33.11	**2388**	**Coumaran**	496-16-2	-	0.32	MS, RI
33.45	**2409**	**δ-Terpineol**	7299-42-5	-	0.58	MS, RI
34.71	**2488**	**Benzophenone**	119-61-9	0.04	0.04	MS, RI
35.68	**2550**	**3-Hydroxy-β-damascone**	102488-09-5	0.06	0.18	MS, RI
36.21	**2585**	**Vanillin**	121-33-5	0.64	0.98	MS, RI
36.56	**2607**	**(*E*)-8-Hydroxygeraniol**	26488-97-1	0.05	0.39	MS, RI
38.66	**2730**	**Cryptomeridiol**	4666-84-6	4.95	5.76	MS, RI
38.80	**2737**	**2,6-Dimethoxy-4-propenylphenol**	20675-95-0	-	0.93	MS, RI
42.33	**2891**	**Hexadecanoic acid**	57-10-3	3.19	10.88	MS, RI
42.69	**2904**	**Syringylaldehyde**	134-96-3	0.45	0.38	MS, RI
43.58	**2934**	**Eugenol**	97-53-0	-	0.14	MS, RI
43.99	**2948**	**α-Copaen-11-ol**	41370-56-3	0.10	0.23	MS, RI
44.18	**2954**	**4-Hydroxy-benzeneethanol**	501-94-0	0.09	7.98	MS, RI
48.26	**3071**	**Stearic acid**	57-11-4	3.38	3.82	MS, RI
51.52	**3147**	**Linoleic acid**	60-33-3	1.36	2.73	MS, RI

52.73	**3173**	**Coniferyl alcohol**	458-35-5	-	0.53	MS, RI

101 volatiles were identified in the peel extract shown in Figure 7 and Table 6, including 54 terpenes, 18 alcohols, 11 aldehydes, 7 acids, 5 esters, 3 phenols, 2 lactones, and 1 N-compound. Major volatiles detected in the peel are limonene (10.53 – 27.85%), *(Z)*-3-hexenol (4.85 – 12.51%), linalool (9.40 – 10.29%), 1-octanol (2.55 – 2.84%), α-terpineol (4.00 – 7.80%), isopiperitenone (1.91%), geraniol (0.79 – 1.06%), 8-hydroxylinalool (1.20 – 2.12%), *(E)*-ρ-mentha-2,8-dien-1-ol (0.39 – 1.61%), and hexadecanoic acid (0.81 – 1.31%), accounting for 35.91 – 67.91% of total volatiles. Aroma characteristics of linalool include floral, waxy, woody and alpha-terpineol is piney and woody.

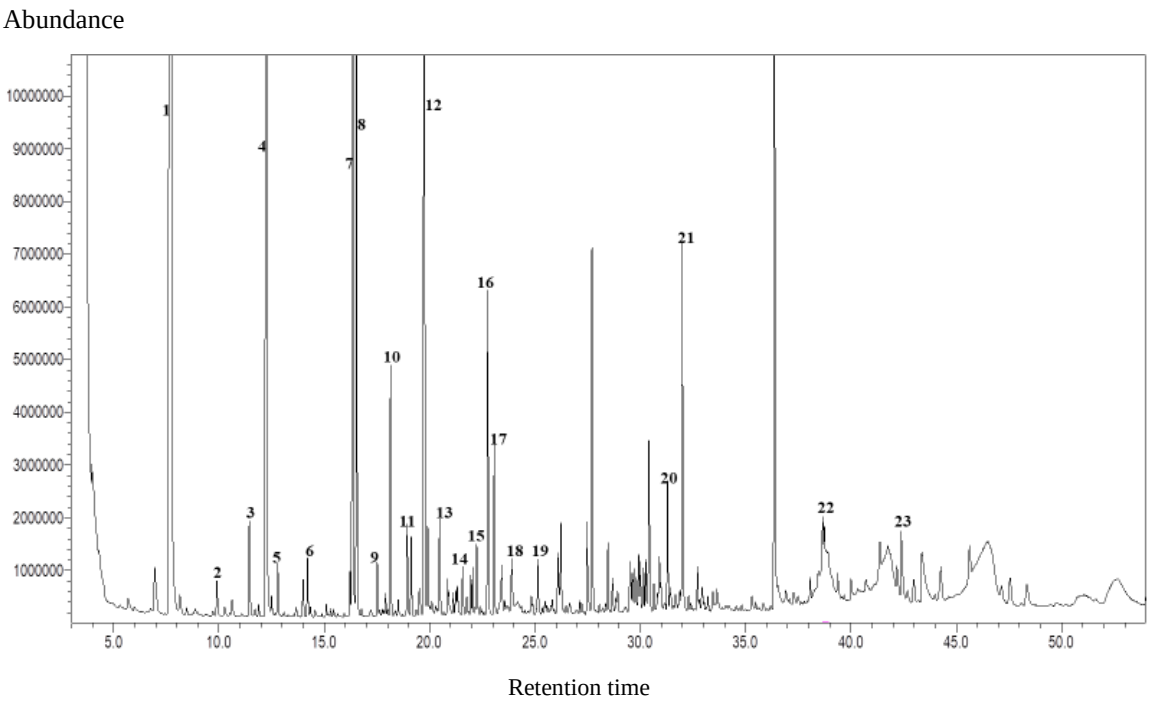

Figure 7. LiChrolut-EN SPE-GC-MS with 95:5 dichloromethane:methanol elution of calamondin peel extract.

Key: (1) limonene (2) octanal (3) hexanol (4) (*Z*)-3-hexenol (5) (*E*)-2-hexenol (6) sabinene hydrate (7) linalool (8) 1-octanol (9) terpinen-4-ol (10) *(E)*-ρ-mentha-2,8-dien-1-ol (11) nonanol (12) α-terpineol (13) d-carvone (14) perilla aldehyde (15) (*E,E*)-2,4-decadienal; (16) isopiperitenone (17) geraniol (18) *(Z)*-ρ-mentha-1(7),8-dien-2-ol (19) 2,6-dimethyl-3,7-octadiene-2,6-diol (20) limonene-diol (21) 8-hydroxylinalool (22) cryptomeridiol (23) hexadecanoic acid.

Table 6

Volatile compounds in SPE with 95:5 dichloromethane:methanol elution from calamondin peel using polar column ZB Wax column (30 m x 0.25 mm x 0.25 µm).

RT (min)	LRI	Compound Name	CAS #	April-Area %	August-Area %	ID Criteria
5.30	**1058**	**Hexanal**	66-25-1	-	0.02	MS, RI
5.70	**1078**	**β-Pinene**	127-91-3	-	0.12	MS, RI
5.99	**1093**	**Sabinene**	3387-41-5	0.03	0.03	MS, RI
6.76	**1128**	**1-Penten-3-ol**	616-25-1	0.02	0.05	MS, RI
6.96	**1136**	**β-Myrcene**	123-35-3	0.17	0.54	MS, RI
7.71	**1169**	**Limonene**	138-86-3	10.53	27.85	MS, RI
8.14	**1188**	**(*E*)-2-Hexenal**	6728-26-3	0.05	0.20	MS, RI
8.78	**1215**	**3-Carene**	13466-78-9	-	0.01	MS, RI
8.88	**1219**	**1-Pentanol**	71-41-0	0.02	0.06	MS, RI
9.39	**1240**	**ρ-Cymene**	99-87-6	-	0.01	MS, RI, S
9.90	**1261**	**Octanal**	124-13-0	0.48	0.31	MS, RI, S
10.27	**1276**	**3-Penten-1-ol**	764-37-4	0.02	0.08	MS, RI
10.63	**1291**	**(*Z*)-2-Hexenol**	928-94-9	-	0.15	MS, RI
11.45	**1324**	**Hexanol**	111-27-3	0.22	0.67	MS, RI, S
12.03	**1347**	**Heptyl acetate**	112-06-1	-	0.01	MS, RI
12.26	**1356**	**(*Z*)-3-Hexenol**	928-96-1	4.85	12.51	MS, RI
12.50	**1366**	**Nonanal**	124-19-6	0.06	0.09	MS, RI
12.70	**1374**	**(*E,E*)-2,4-Hexadienal**	142-83-6	-	0.01	MS, RI
12.79	**1378**	**(*E*)-2-Hexenol**	928-95-0	0.08	0.32	MS, RI
13.35	**1400**	**(*E*)-2-Octenal**	2548-87-0	-	0.01	MS, RI
13.68	**1414**	**Limonene oxide**	1195-92-9	-	0.08	MS, RI

14.00	**1427**	**Isopinocampheol**	27779-29-9	-	0.24	MS, RI
14.21	**1436**	**Sabinene hydrate**	546-79-2	0.49	0.33	MS, RI
14.34	**1442**	***(E)*-Linalool oxide**	34995-77-2	-	0.07	MS, RI
14.56	**1452**	**Octyl acetate**	112-14-1	0.02	0.05	MS, RI
14.88	**1465**	**2-Ethylhexanol**	104-76-7	-	0.02	MS, RI
15.10	**1475**	**Decanal**	112-31-2	0.04	0.08	MS, RI, S
15.32	**1484**	**Camphor**	464-49-3	-	0.05	MS, RI
15.64	**1498**	**Benzaldehyde**	100-52-7	-	0.01	MS, RI
15.95	**1511**	**(*E*)-2-Nonenal**	18829-56-6	-	0.03	MS, RI
16.10	**1518**	**(*E*)-4-Decenal**	65405-70-1	-	0.01	MS, RI
16.22	**1523**	**(*E*)-Sabinene hydrate**	17699-16-0	-	0.24	MS, RI
16.35	**1529**	**Linalool**	78-70-6	10.29	9.40	MS, RI, S
16.54	**1537**	**1-Octanol**	111-87-5	2.55	2.84	MS, RI
16.77	**1547**	**β-Copaene**	18252-44-3	-	0.05	MS, RI
17.03	**1558**	**Nonyl acetate**	143-13-5	-	0.00	MS, RI
17.22	**1566**	**β-Elemene**	33880-83-0	-	0.03	MS, RI
17.52	**1579**	**Terpinen-4-ol**	562-74-3	0.14	0.27	MS, RI, S
17.62	**1584**	***(Z)*-Dihydrocarvone**	3792-53-8	-	0.05	MS, RI
17.75	**1590**	***(E)*-β-Ocimene**	3779-61-1	-	0.05	MS, RI
17.83	**1593**	**(*E*)-2-Octen-1-ol**	18409-17-1	-	0.03	MS, RI
18.02	**1601**	***(E)*-Dihydrocarvone**	5948-04-9	-	0.07	MS, RI
18.15	**1607**	***(E)*-ρ-Mentha-2,8-dien-1-ol**	7212-40-0	0.39	1.61	MS, RI

18.37	**1617**	**α-Santalene**	512-61-8	-	0.05	MS, RI
18.51	**1623**	**(*E*)-2-Decenal**	3913-81-3	-	0.16	MS, RI
18.87	**1640**	**γ-Muurolene**	30021-46-6	-	0.01	MS, RI
18.94	**1643**	**Nonanol**	143-08-8	0.14	0.53	MS, RI
19.37	**1662**	**Citral**	106-26-3	0.04	0.05	MS, RI, S
19.43	**1665**	**Decyl acetate**	112-17-4	-	0.01	MS, RI
19.52	**1669**	***(Z)*-Carveol**	1197-06-4	-	0.21	MS, RI
19.75	**1680**	**α-Terpineol**	98-55-5	4.00	7.80	MS, RI, S
19.91	**1687**	**Isogermacrene D**	317819-80-0	-	0.55	MS, RI
20.11	**1696**	**β-Selinene**	17066-67-0	0.07	0.12	MS, RI
20.29	**1704**	**Piperitone**	89-81-6	-	0.08	MS, RI
20.38	**1708**	**Neodihydrocarveol**	18675-33-7	-	0.04	MS, RI
20.48	**1713**	**d-Carvone**	2244-16-8	0.27	0.60	MS, RI, S
20.85	**1731**	***(Z)*-Isopiperitenol**	96555-02-1	0.06	0.20	MS, RI
20.96	**1736**	***(E)*-Isopiperitenol**	74410-00-7	0.02	0.06	MS, RI
21.13	**1744**	**Geranyl acetate**	105-87-3	0.07	0.13	MS, RI, S
21.24	**1749**	**Decanol**	112-30-1	-	0.18	MS, RI
21.32	**1753**	**Citronellol**	106-22-9	0.06	0.17	MS, RI, S
21.39	**1756**	**Methyl salicylate**	119-36-8	-	0.02	MS, RI
21.57	**1765**	**Perilla aldehyde**	2111-75-3	0.11	0.28	MS, RI
21.85	**1778**	**2-Decen-1-Ol**	18409-18-2	-	0.03	MS, RI
21.96	**1783**	***(E)*-ρ-Mentha-1(7),8-dien-2-ol**	21391-84-4	-	0.18	MS, RI
22.05	**1787**	**Nerol**	106-25-2	0.10	0.38	MS, RI

22.23	**1796**	***(E,E)*-2,4-Decadienal**	25152-84-5	0.09	0.41	MS, RI
22.30	**1799**	**Citronellal**	106-23-0	-	0.02	MS, RI
22.59	**1814**	***(E,E)*-2,6-Dimethyl-3,5,7-octatriene-2-ol**	29414-56-0	-	0.02	MS, RI
22.77	**1822**	**Isopiperitenone**	529-01-1	-	1.91	MS, RI
23.07	**1837**	**Geraniol**	106-24-1	0.79	1.06	MS, RI, S
23.25	**1846**	**Hexanoic acid**	142-62-1	-	0.01	MS, RI, S
23.66	**1866**	**Benzyl alcohol**	100-51-6	-	0.07	MS, RI
23.90	**1878**	***(Z)*-ρ-Mentha-1(7),8-dien-2-ol**	22626-43-3	0.06	0.44	MS, RI
24.35	**1901**	**Phenylethyl alcohol**	60-12-8	0.02	0.06	MS, RI
24.44	**1905**	**Piperitenone**	491-09-8	-	0.02	MS, RI
24.89	**1928**	**ρ-Menth-1-en-9-ol**	18479-68-0	0.03	0.18	MS, RI
25.14	**1941**	**2,6-Dimethyl-3,7-octadiene-2,6-diol**	13741-21-4	0.08	0.23	MS, RI
25.55	**1962**	**Dodecanol**	112-53-8	-	0.06	MS, RI
25.63	**1967**	**Heptanoic acid**	111-14-8	-	0.05	MS, RI
25.92	**1981**	***(E,E)*-2,4-Decadienol**	18409-21-7	-	0.03	MS, RI
26.09	**1990**	**Limonen-10-ol**	38142-45-9	0.15	0.46	MS, RI
26.23	**1998**	**Perilla alcohol**	536-59-4	0.32	0.58	MS, RI, S
26.36	**2004**	**Phenol**	108-95-2	0.03	0.02	MS, RI
28.23	**2105**	**γ-Decalactone**	706-14-9	-	0.06	MS, RI
28.68	**2130**	**2,6-Dimethyl-1,7-octadiene-3,6-diol**	51276-33-6	0.08	0.15	MS, RI
29.74	**2189**	**Nonanoic acid**	112-05-0	0.12	0.37	MS, RI

29.92	**2199**	**2-Methoxy-4-vinylphenol**	7786-61-0	1.23	0.38	MS, RI
30.91	**2257**	**Jasmine lactone**	25524-95-2	0.03	0.47	MS, RI
31.30	**2279**	**Limonene-diol**	1946-00-5	0.30	0.72	MS, RI
31.67	**2301**	**Decanoic acid**	334-48-5	0.03	0.18	MS, RI
31.99	**2320**	**8-Hydroxylinalool**	64142-78-5	1.20	2.12	MS, RI
33.55	**2415**	**Isoelemicin**	487-12-7	-	0.05	MS, RI
34.20	**2456**	**Indole**	120-72-9	-	0.10	MS, RI
36.19	**2584**	**Vanillin**	121-33-5	-	0.05	MS, RI
36.68	**2614**	**8-Hydroxygeraniol**	26488-97-1	-	0.03	MS, RI
37.28	**2651**	**Perillic acid**	7694-45-3	0.19	0.13	MS, RI
38.66	**2730**	**Cryptomeridiol**	4666-84-6	0.26	0.69	MS, RI
42.39	**2894**	**Hexadecanoic acid**	57-10-3	1.31	0.81	MS, RI
48.36	**3074**	**Stearic acid**	57-11-4	0.19	0.43	MS, RI
51.64	**3149**	**Linoleic acid**	60-33-3	-	0.10	MS, RI

Analysis of the juice and peel from harvests in April and August varied in compounds and their % peak area. Comparison of the juice from both harvests shared the following 43 compounds: myrcene, limonene, 4,8-dimethyl-1,3,7-nonatriene, prenol, (*Z*)-3-hexenol, 2-ethyl-1-hexanol, benzaldehyde, linalool, 1-octanol, fenchol, terpinen-4-ol, 1-nonanol, decyl acetate, γ-muurolene, α-terpineol, α-amorphene, carvone, isopiperitenol, α-muurolene, decanol, isopiperitenone, geraniol, hexanoic acid, ρ-menth-1-en-9-ol, dodecanol, 2-pyrrolidinone, viridiflorol, α-cadinol, intermedeol, limonen-1,2-diol, (*Z*)-8-hydroxylinalool, (*E*)-isoeugenol, benzophenone, 3-hydroxy-β-damascone, vanillin, (*E*)-8-

hydroxygeraniol, cryptomeridiol, hexadecanoic acid, syringylaldehyde, α-copaen-11-ol, 4-hydroxy-benzeneethanol, stearic acid, and linoleic acid. The April harvest of the 43 compounds account for 42.89% of total volatiles compared to the August harvest at 69.76%. Significant differences in concentrations were in compounds (*Z*)-8-hydroxylinalool in the April harvest at 0.45% and 3.58% in the August harvest, hexadecanoic acid in the April harvest at 3.19% and 10.88% in the August harvest, and 4-hydroxy-benzeneethanol in the April harvest at 0.09% and 7.98% in the August harvest. Higher concentrations of myrcene, benzaldehyde, linalool, 1-octanol, fenchol, terpinen-4-ol, 1-nonanol, decyl acetate, α-amorphene, geraniol, viridiflorol, α-cadinol, intermedeol, and syringylaldehyde were found in the April harvest than August harvest. Limonene, 4,8-dimethyl-1,3,7-nonatriene, prenol, (*Z*)-3-hexenol, 2-ethyl-1-hexanol, γ-muurolene, α-terpineol, carvone, isopiperitenol, α-muurolene, decanol, isopiperitenone, hexanoic acid, dodecanol, 2-pyrrolidinone, limonen-1,2-diol, (*E*)-isoeugenol, 3-hydroxy-β-damascone, vanillin, (*E*)-8-hydroxygeraniol, cryptomeridiol, α-copaen-11-ol, stearic acid, and linoleic acid showed a lower concentration level in April harvest compared to August harvest while the concentrations of ρ-menth-1-en-9-ol and benzophenone shared the same concentration for both harvests.

Comparison of the peel from both harvests shared the following 50 compounds: sabinene, 1-penten-3-ol, β-myrcene, limonene, (*E*)-2-hexenal, 1-pentanol, octanal, 3-penten-1-ol, hexanol, (*Z*)-3-hexenol, nonanal, (*E*)-2-hexenol, sabinene hydrate, octyl acetate, decanal, linalool, 1-octanol, terpinen-4-ol, *(E)*-ρ-mentha-2,8-dien-1-ol, nonanol,

citral, α-terpineol, β-selinene, d-carvone, *(Z)*-isopiperitenol, *(E)*-isopiperitenol, geranyl acetate, citronellol, perilla aldehyde, nerol, *(E,E)*-2,4-decadienal, geraniol, *(Z)*-ρ-mentha-1(7),8-dien-2-ol, phenylethyl alcohol, ρ-menth-1-en-9-ol, 2,6-dimethyl-3,7-octadiene-2,6-diol, limonen-10-ol, perilla alcohol, phenol, 2,6-dimethyl-1,7-octadiene-3,6-diol, nonanoic acid, 2-methoxy-4-vinylphenol, jasmine lactone, limonene-diol, decanoic acid, 8-hydroxylinalool, perillic acid, cryptomeridiol, hexadecanoic acid, and stearic acid. The April harvest of the 50 compounds account for 41.90% of total volatiles compared to the August harvest at 77.50%. Significant difference in quantities were in compounds limonene in the April harvest at 10.53% and 27.85% in the August harvest, (*Z*)-3-hexenol in the April harvest at 4.85% and 12.51% in the August harvest, and α-terpineol in the April harvest at 4.00% and 7.80% in the August harvest. Higher concentrations of octanal, sabinene hydrate, linalool, phenol, 2-methoxy-4-vinylphenol, perillic acid, and hexadecanoic acid were found in the April harvest than August harvest. 1-penten-3-ol, β-myrcene, (*E*)-2-hexenal, 1-pentanol, 3-penten-1-ol, hexanol, nonanal, (*E*)-2-hexenol, octyl acetate, decanal, 1-octanol, terpinen-4-ol, *(E)*-ρ-mentha-2,8-dien-1-ol, nonanol, citral, β-selinene, d-carvone, *(Z)*-isopiperitenol, *(E)*-isopiperitenol, geranyl acetate, citronellol, perilla aldehyde, nerol, *(E,E)*-2,4-decadienal, geraniol, *(Z)*-ρ-mentha-1(7),8-dien-2-ol, phenylethyl alcohol, ρ-menth-1-en-9-ol, 2,6-dimethyl-3,7-octadiene-2,6-diol, limonen-10-ol, perilla alcohol, 2,6-dimethyl-1,7-octadiene-3,6-diol, nonanoic acid, jasmine lactone, limonene-diol, decanoic acid, 8-hydroxylinalool, cryptomeridiol, and stearic acid showed

lower concentration in April harvest compared to August harvest while the concentration of sabinene shared the same concentration for both harvests.

Quantitative Descriptive Analysis

QDA consisted of 12 trained panelists evaluating the smell of the zest, the smell of the juice, and the taste of the juice to rate the intensity of each descriptor on an instructed line scale (0-10). A total of 19 sensory attributes on calamondin peel and juice were generated including 14 aroma descriptors: fresh, fatty, green, peely, waxy, fruity, juicy, lime, piney, floral, woody, mandarin, grapefruit, sweet; and 5 taste descriptors: acidic, sourness, astringent, bitter, and salivating.

Preparation of Standards

Descriptor standards used for training the panel were prepared with propylene glycol (PG) stock solutions and reference chemicals according to Table 1. Some references such as fresh, fatty, green, waxy, fruity, piney, floral, woody, mandarin, grapefruit, acidic, and sweet are formulated using a single chemical, while the other references of peely, juicy, and lime are a combination of multiple chemicals. The chemicals were selected based on the volatile analysis data, literature, and the thesis chair's experience. The dose for each reference was formulated by trial-and-error. The perceived intensity of each standard had been determined as 5 and agreed upon by the panel, using continuous 11-point unstructured line scale from 0 to 10.

Sensory Evaluation of Calamondin Peel (Zest) and Juice

Mean scores and standard deviations (SD) are shown in Table 7. Overall, the perceived intensity for the attributes of calamondin peel and juice was ranged an intensity of 2 – 7 on a 0 – 10 intensity scale, which was a good spread. Most intensive attributes indicated by their high levels of intensity for calamondin peel smell were peely at 6.8, fresh at 5.7, and fatty at 5.3. For the juice smell, most intense attributes were juicy at 5.8, acidic at 5.7, and fresh at 5.2. Intense attributes for the taste of the juice were sourness at 8.9, salivating at 7.8, and astringent at 7.5. Overall, the intensities of the aroma attributes fatty, peely, waxy, piney, floral, woody, and sweet differed significantly ($p < 0.05$), indicating a strong relation among these attributes across the three samples.

Table 7

Mean scores and SD of descriptive sensory analysis of newly harvested, immature calamondin. Different letters mean significant difference within each descriptor across different sample by one-way ANOVA Tukey's HSD test, $p < 0.05$.

	Zest Smell			Juice Smell			Juice Taste		
	Mean score	**SD**	**Difference**	**Mean score**	**SD**	**Difference**	**Mean score**	**SD**	**Difference**
Fresh	5.7	2.6	a	5.2	2.2	a	5.2	2.6	a
Fatty* **	5.3	1.9	b	3.3	2.2	a	3.5	1.9	a
Green	4.8	2.8	a	4.5	2.6	a	4.6	2.3	a
Peely* **	6.8	1.9	b	4.2	2.5	a	5.2	2.5	a
Waxy* **	4.9	2.2	b	2.8	2.1	a	3.7	2.2	a
Fruity	3.3	2.4	a	3.9	2.4	a	3.7	2.4	a
Juicy	4.7	2.3	a	5.8	2.5	a	5.6	2.6	a
Lime	4.8	2.2	a	3.9	2.4	a	4.7	2.4	a
Piney **	4.7	2.5	b	3.1	2.6	a	4.2	2.4	a,b
Floral*	4.2	2.4	b	3.2	2.2	a	3.2	2.8	a
Woody*	3.8	2.5	b	2.5	2.3	a	3.4	2.8	b
Mandarin	4.4	2.4	a	5.5	2.3	a	4.8	2.2	a

Grapefruit	4.1	2.1	a	4.4	2.4	a	4.7	2.3	a
Sweet* **	3.7	2.7	b	2.9	1.5	a,b	1.9	1.4	a
Acidic*	4.6	2.8		5.7	2.8				
Sourness							8.9	0.8	
Astringent							7.5	1.8	
Bitter							5.4	2.7	
Salivating							7.8	1.8	

The spider chart in Figure 8 shows that peely being the most intense attribute in the aroma of the zest with an intensity at 6.8, followed by fresh at 5.7, fatty at 5.3, waxy at 4.9, green at 4.8, lime at 4.8, juicy at 4.7, piney at 4.7, and acidic at 4.6. Less intense attributes include fruity with an intensity at 3.3, floral (orange flower) at 4.2, woody at 3.8, mandarin at 4.4, grapefruit at 4.1, and sweet at 3.7.

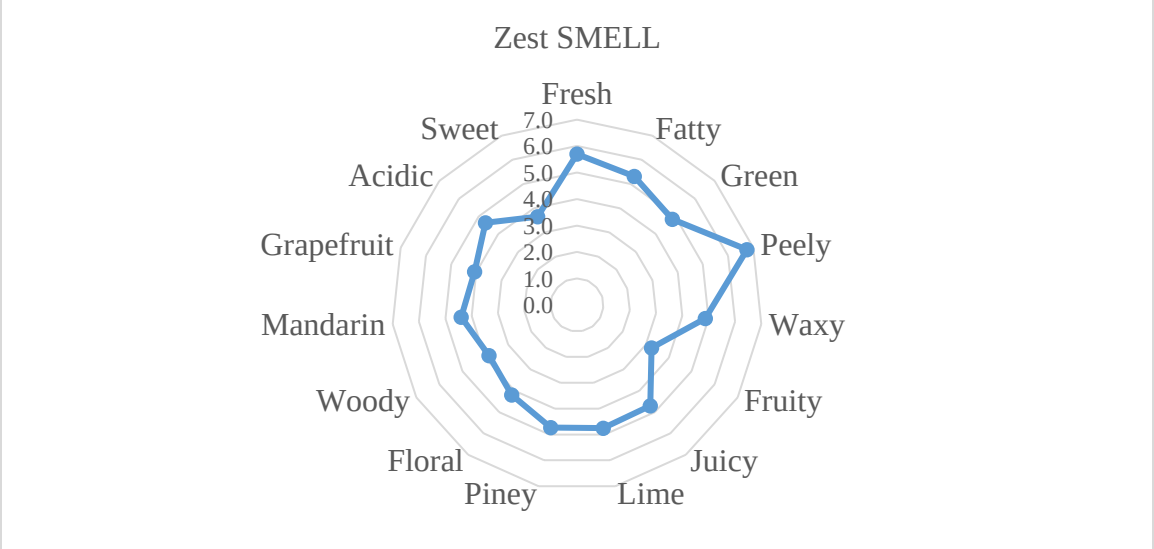

*Figure 8***.** Spider plot of the mean scores of descriptive sensory evaluation for the smell of the calamondin zest.

The spider chart in Figure 9 shows that the aroma of the juice has the most intense attributes of juicy with intensity at 5.8, acidic at 5.7, mandarin at 5.5, fresh at 5.2, green at 4.5, grapefruit at 4.4, and peely at 4.2. Less intense attributes include fatty at 3.3, waxy at 2.8, fruity at 3.9, lime at 3.9, piney at 3.1, floral (orange flower) at 3.2, woody at 2.5, and sweet at 2.9.

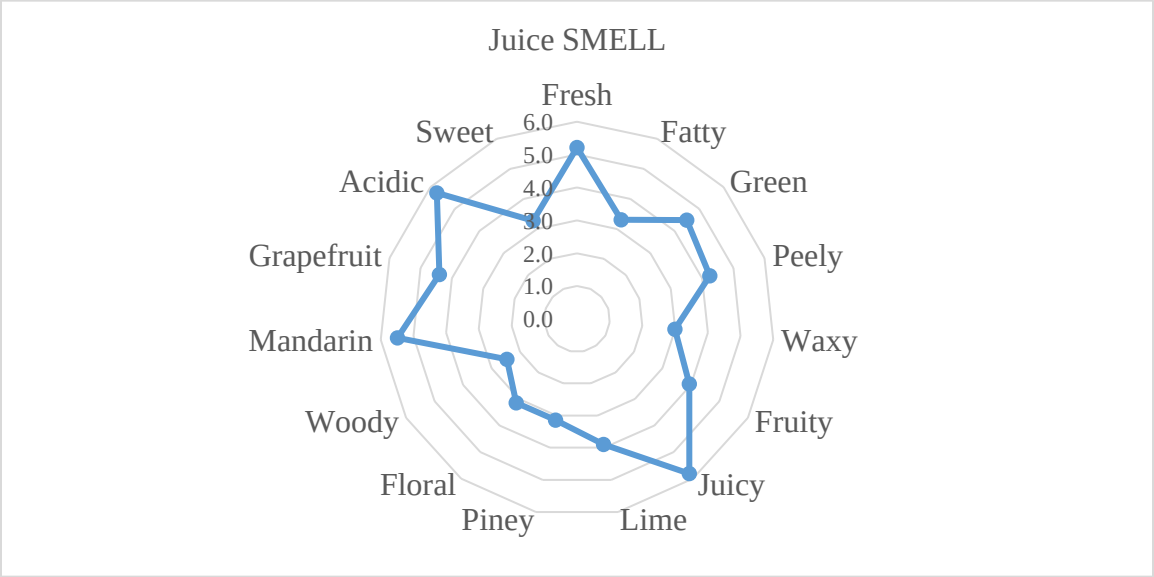

Figure 9. Spider plot of the mean scores of descriptive sensory evaluation for the smell of the calamondin juice.

The spider chart in Figure 10 shows that the taste of the juice has the most intense attributes of sourness with intensity at 8.9, salivating at 7.8, and astringent at 7.5. Moderately intense attributes include juicy with intensity at 5.6, fresh at 5.2, peely at 5.2, bitter at 5.4, green at 4.6, lime at 4.7, piney at 4.2, mandarin at 4.8, and grapefruit at 4.7. Less intense attributes are fatty at 3.5, waxy at 3.7, fruity at 3.7, floral (orange blossom) at 3.2, woody at 3.4, and sweet at 1.9.

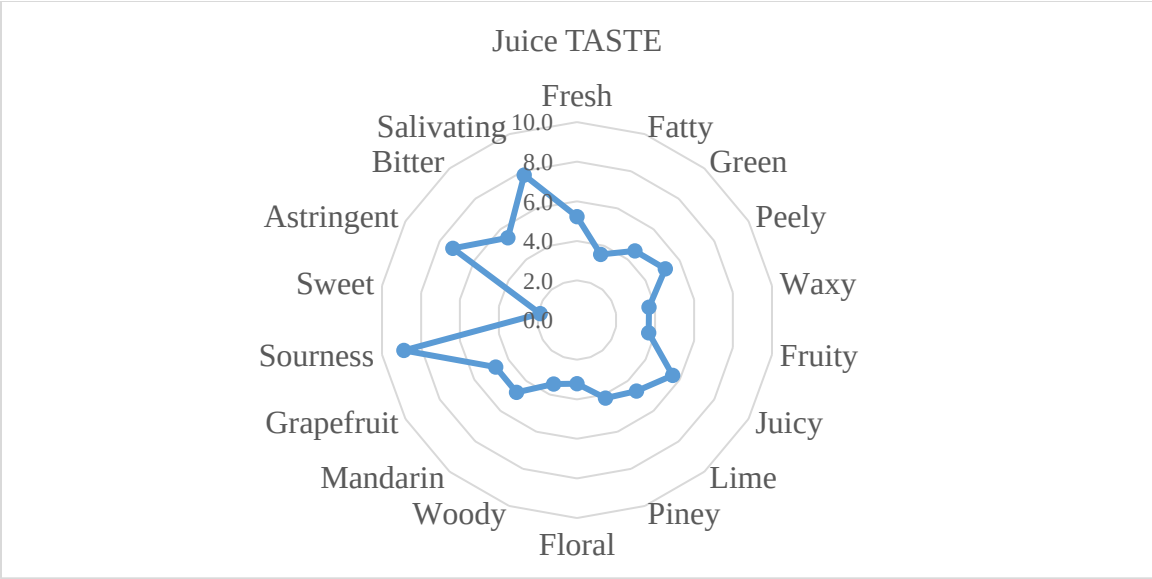

Figure 10. Spider plot of the mean scores of descriptive sensory evaluation for the taste of the calamondin juice.

Principal Component Analysis

PCA correlation analysis was performed to investigate the relationship between sensory descriptors and three calamondin samples (see Figure 11), using the mean values of each descriptor's intensity for each calamondin sample when the panelists were asked about the aroma of the calamondin samples. The loading values of the 14 aroma descriptors were used to calculate the score values for the three calamondin samples. The PC1 axis explains approximately 83.92% of the variance alone, while PC2 accounts for 16.08%. It means PC1 is the major component to differentiate samples by their descriptors. Calamondin peel was separated on the left-hand side of the plot corresponding to negative PC1 values, while calamondin juice smell and taste were clustered on the right-hand side of the plot corresponding to positive PC1 values. Calamondin zest smelled highly in green, waxy, peely, fatty, fresh, woody, piney, and lime notes. Calamondin juice smelled highly

in fruity, juicy, and mandarin notes, while calamondin juice taste had a grapefruit-like aroma.

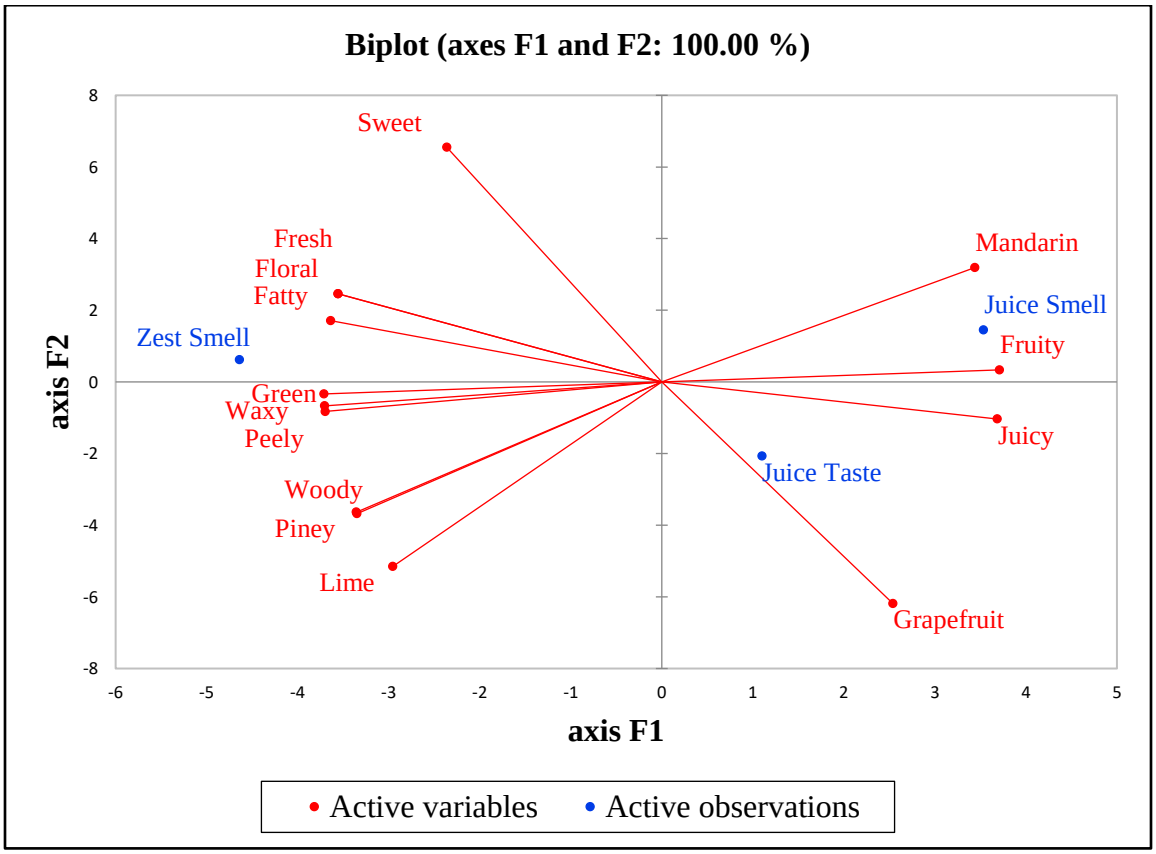

Figure 11. Principle Component Analysis (PCA) of three calamondin samples as loading values and all 15 aroma attributes as score values.

CHAPTER V

DISCUSSION

ºBrix, pH, and Titratable Acidity

It is well documented that the stage of fruit ripening impacts characteristics such as size, shape, pigment, taste, flavor, and aroma synthesis; in particular, the emission of volatile components evolves in order to protect immature fruits from pests and herbivores (Marzocchi, Baldi, Crucitti, Toselli, & Caboni 2019). In addition to the volatiles, the aroma and taste of citrus juice also depend on the balance between sugars and organic acids which are among the major non-volatiles (Cheong et al., 2012b).

Physicochemical properties of ºBrix, pH, and TA play a significant role in these characteristics, especially the sweet and sour taste of fruits. The only publications to date that have documented these physicochemical properties of calamondin are when the fruit is in its orange-mature stage, with ºBrix values ranging from 7.6-8.1, pH values ranging from 2.50-2.57, and TA values ranging from 5.66-6.14% (Cheong et al., 2012b; Nisperos-Carriedo et al., 1992). In comparison to the immature calamondin fruit used in this study, the ºBrix value of the harvest date in April was similar in range with the orange-mature stage, yet the value for the harvest date in August was higher at 10.1. Since ºBrix value is a measurement of total soluble solids, the high ºBrix of calamondin juice implies a higher sucrose content and other potential solids such as soluble pectin. The same trend was

observed for organic acid content, the higher value in the August harvest suggested an increase of organic acids, particularly citric acid since TA measured considerably high at 8.72. The value measuring higher in the green-mature calamondin is synonymous with its characteristic sour flavor compared to the orange-mature stage with a distinctive less sour and sweeter flavor (Aggie Horticulture, n.d.). The pH values measured for both harvests of the green-immature fruit were similar in range to the orange-mature fruit indicating pH is not significantly impacted by the seasonal variations and ripening stages (Cheong et al., 2012b). The pH value of the juice may not be directly related to its titratable acidity as pH is only a measurement of free hydrogen ion activity while TA measures the total acid concentration (Cheong et al., 2012b).

Volatile Isolation Method Development

SPME is a commonly used flavor extraction method, that has been performed on calamondin (Cheong et al., 2012b; Nisperos-Carriedo et al., 1992; Takeuchi et al., 2005; Yamamoto et al., 2012; Yo et al., 2004). Since optimization of a volatile profile incorporates applying and analyzing numerous extraction methods for variety of compounds, SPME analysis was done on the calamondin juice and zest for volatile comparison to SPE data.

SPME-GC-MS Calamondin Juice Volatiles

Volatiles identified in the juice from immature calamondin fruit were compared to reported volatiles in the juice by SPME analysis (Cheong et al., 2012b; Nisperos-Carriedo et al., 1992; Yamamoto et al., 2012; Yo et al., 2004). Current literature indicates that SPME

analysis of the juice was from fruit in the mature stage, or the maturity of the fruit was not stated at all (Cheong et al., 2012b; Nisperos-Carriedo et al., 1992; Yamamoto et al., 2012; Yo et al., 2004). Reported volatiles identified in the juice state that the juice was prepared by manually squeezing fruit not initially peeled, or the literature did not specify if the fruit was peeled (Nisperos-Carriedo et al., 1992; Yamamoto et al., 2012; Yo et al., 2004). Due to the incorporation of peel oil in the juice during preparation, it resulted in the intensified flavor of citrus juice, but altered its original aroma profile (Bazemore, Goodner, & Rouseff, 1999). Studies have shown that the level of volatiles in citrus increased until fruit reached maturity (Barboni et al., 2009). Sweetness, ripeness, and fruity flavor increased with fruit maturity (Hijaz et al., 2020). In a study on the effect of fruit maturity on volatiles of mandarin hybrids, monoterpenes tended to decrease with fruit maturity, whereas alcohols, esters, and aldehydes tended to increase (Hijaz et al., 2020). Although these reports identified differences in the fruit maturity compared to the immature fruit, all major volatiles identified in this study have been reported (Cheong et al., 2012b; Nisperos-Carriedo et al., 1992; Yamamoto et al., 2012; Yo et al., 2004).

Terpene hydrocarbons are known to be the major components of citrus essential oils that contribute to characteristic citrusy and woody notes (Cheong et al., 2012b). Previous studies have identified limonene, germacrene D, β-myrcene, and β-cymene as dominant components of calamondin juice (Cheong et al., 2012b; Nisperos-Carriedo et al., 1992; Yamamoto et al., 2012; Yo et al., 2004). It is also suggested that β-selinene and limonene, together with small amounts of oxygenated terpenes, are responsible for the

aroma of calamondin fruit (Moshonas et al., 1996). Of the 123 volatiles identified in the juice by SPME analysis, 48 were similar to those identified in the literature, which includes all of the major volatiles: ethanol, β-pinene, β-myrcene, limonene, 4,8-dimethyl-1,3,7-nonatriene, δ-cadinene, and germacrene D. Limonene, which has the highest concentration of all the volatiles, imparts a citrus, herbal, and camphor aroma and is common in citrus fruits (The Good Scents Company, 2018). Ethanol imparts an alcoholic, ethereal, and medicinal aroma, commonly occurring in citrus fruit; β-pinene is characterized by fresh, piney, and woody aroma, and a slight minty, camphoraceous with a spicy nuance flavor; β-myrcene is described as having a peppery, spicy aroma, and a flavor of woody, citrus, and fruity with a tropical mango and slight leafy, minty, nuances; δ-cadinene induces a thyme, herbal, and woody aroma; germacrene D gives a woody, earthy, and spicy aroma (The Good Scents Company, 2018). The 4,8-dimethyl-1,3,7-nonatriene is reported to be one of the major compounds responsible for the floral scent of the "Sunny Bell" *Cymbidium* flower and has also been identified as a component of cardamom oil (Baek et al., 2019; Maurer, Hauser, & Froidevaux 1986).

The compound 4,8-dimethyl-1,3,7-nonatriene is also known to be one of the major components in the blend of volatiles produced by cucumber (*Cucumis sativus* L.), lima bean (*Phaseolus lunatus* L.), and many different plant species in response to herbivory by insects and spider mites (Bouwmeester, Verstappen, Posthumus, & Dicke 1999). The calamondin used in this study was analyzed in its immature stage, so although this

compound may not directly contribute to its aroma profile, it contributes to the fruit's protection from pests and herbivores (Marzocchi et al., 2019).

Other volatiles identified, including acetoin (sweet, buttery, creamy aroma with a sweet, oily, milky flavor), 1-hexanol (fruity, alcoholic, green aroma with a fruity, apple skin flavor), 2-ethyl-1-hexanol (citrus, fresh, floral aroma with a sweet, fatty, fruity flavor), benzaldehyde (sharp, bitter almond aroma with an oily, nutty, and woody flavor), nonanol acetate (waxy, green, tropical fruit aroma with a fruity, waxy and tropical fruit flavor), carvone (minty, licorice aroma), citronellol (waxy, rose bud, citrusy aroma with a flavor of rose, green with fruity citrus nuances), nerol (sweet, citrus, magnolia aroma with a flavor of bitter, green, and fruity with terpy nuances), geraniol (rose, waxy, citrusy aroma with a flavor of rosy, waxy and perfumey with a fruity, peach-like nuance), and 10-epi-γ-eudesmol (sweet, woody, and floral aroma), have been reported in the juice but were not extracted using SPME analysis, rather using solvent extraction (Takeuchi et al., 2005; The Good Scents Company, 2018). Additionally, identified volatiles from the juice are similar to reported volatiles extracted from the peel, peel oil, and/or leaf oil by solvent, distillation, and/or SDE methods: camphene (peel, leaf oil), α-phellandrene (peel, peel oil, leaf oil), *(E)*-β-ocimene (peel), *(Z)*-3-hexenol acetate (peel), 1-hexanol (peel, juice, peel oil, leaf oil), 1,3,8-ρ-menthatriene (leaf oil), α-cubebene (peel, peel oil), camphor (peel), β-copaene (peel oil, leaf oil), valencene (peel), γ-muurolene (leaf oil), carvone (peel, juice, peel oil, leaf oil), 1-decanol (peel), citronellol (peel, juice, peel oil, leaf oil), nerol (peel, juice, peel oil, leaf oil), geraniol (peel, juice), *(E)*-nerolidol (peel), and 10-epi-γ-eudesmol (peel, juice,

peel oil, leaf oil; Chen et al., 2013; Cheong et al., 2012a; Cheong et al., 2012b; Cuevas-Glory et al., 2009; Moshonas et al., 1996; Takeuchi et al., 2005; Yamamoto et al., 2012; Yo et al., 2004).

Apart from the components reported in previous studies, 52 volatiles were reported here for the first time (Cheong et al., 2012b; Nisperos-Carriedo et al., 1992; Yamamoto et al., 2012; Yo et al., 2004). The newly identified volatiles included α-cadinene (woody, dry aroma), α-cadinol (herbal, woody aroma), α-elemene (floral aroma), cedrol (cedarwood, sweet aroma with an amber, floral, and musk flavor), δ-cadinol (floral aroma), cosmene (floral aroma), τ-muurolol (herbal, spicy, honey aroma), selina-3,7(11)-dien (herbal, woody aroma), *(E)*-calamenene (herbal, spicy aroma), *(Z)*-calamenene (herbal, spicy aroma), junenol, viridiflorol (green, herbal, tropical fruity, minty aroma), neointermedeol, butyl acetate (ethereal, fruity, banana aroma with a flavor of sweet, tropical and candy-like with green nuances), ρ-mentha-1-en-9-ol (fruity, herbal aroma with a herbal flavor), isopropenyl methyl ketone, isopropenyl ethyl ketone, *(E)*-3-penten-2-one (fruity, acetone, fishy aroma with a musty, fishy flavor), 2-heptanone (fruity, herbal, coconut aroma with a green, waxy, coconut, cheese flavor), 2-nonanone (fresh, green, herbal aroma with a cheesy, dairy, buttery flavor), 1-octen-3-ol (mushroom, earthy, green aroma with a mushroom, earthy, umami sensation flavor), isovaleric aldehyde (chocolate, peach, fatty aroma with a fruity, green, chocolate, nutty flavor), isopropyl alcohol (alcohol, musty, woody aroma with an alcoholic, woody, musty flavor), 2,4-dimethylfuran, α-fenchene (camphoreous aroma), dehydrosabinene, ethylbenzene, *ρ*-xylene, 1-butanol (sweet,

balsamic, whiskey aroma with a fruity, banana flavor), 4-carene (piney, musky, earthy aroma), *(E)*-2-hexenal (green, banana, fatty, cheesy aroma with a fresh, green, fruity flavor), 2-pentylfuran (fruity, green, earthy, beany aroma with a waxy, musty, cooked caramellic flavor), *(Z)*-2-heptenal (green, fatty aroma), sulcatone (citrus, musty, apple aroma with a green, vegetable, banana flavor), rose oxide (green, rosy, fresh, floral aroma with a citrus, herbal, vegetable flavor), 2-bornene, 2,3-butanediol (fruity, creamy, buttery aroma), fenchol (pine, woody, sweet, lemon aroma with a camphoreous, cooling, minty flavor), sibirene, bicyclosesquiphellandrene, α-muurolene (woody aroma), *(Z)*-carvyl acetate (green, spearmint, fruity aroma with a spearmint, herbal flavor), cubenene (spicy, fruity, mango aroma), α-isomethyl ionone, *(E)*-geranylacetone (fresh, fruity, tropical, rose aroma with a floral, pear, green flavor), hexanoic acid (sour, fatty, cheesy aroma with a cheesy, fruity, fatty flavor), ethylhexanoic acid, 1-dodecanol (soapy, waxy, fatty aroma with an earthy, cilantro, fatty flavor), cedrelanol (balsamic, earthy aroma), hexyl salicylate (fresh, herbal, green aroma with a herbal, green, metallic flavor), and isospathulenol (Botanica Testing Inc, 2019; The Good Scents Company, 2018). Although these volatiles were identified in calamondin juice for the first time, they have been reported in other citrus fruits (González-Mas, Rambla, López-Gresa, Blázquez, & Granell 2019).

SPME-GC-MS Calamondin Peel Volatiles

To date, there are no reports on SPME analysis of the peel. Of the 83 volatiles identified in the zest, 56 are similar to previously reported volatiles found in the peel extracted by solvent or distillation methods (Chen et al., 2013; Cheong et al., 2012a;

Cuevas-Glory et al., 2009; Moshonas et al., 1996; Takeuchi et al., 2005). The identified volatiles included acetaldehyde, α-pinene, β-pinene, sabinene, β-myrcene, limonene, *(E)*-β-ocimene, γ-terpinene, terpinolene, octanal, *(Z)*-3-hexenyl acetate, hexanol, *(Z)*-3-hexenol, nonanal, *(Z)*-limonene oxide, *(E)*-limonene oxide, δ-elemene, octyl acetate, α-copaene, decanal, β-bourbonene, β-cubebene, linalool, 1-octanol, nonanol acetate, β-elemene, β-caryophyllene, terpinen-4-ol, undecanal, *(E)*-2-decenal, 1-nonanol, citral, decyl acetate, α-terpineol, germacrene D, β-selinene, bicyclogermacrene, geranyl acetate, perillaldehyde, γ-cadinene, *(E,E)*-2,4-decadienal, isopeperitenone, geraniol, *(E)*-2-dodecenal, *(E,Z)*-2,6-dodecadienal, perillyl acetate, perillyl alcohol, *(E)*-nerolidol, elemol, γ-eudesmol, α-eudesmol, β-eudesmol, limonene-1,2-diol, indole, and hexadecanoic acid (Chen et al., 2013; Cheong et al., 2012a; Cuevas-Glory et al., 2009; Moshonas et al., 1996; Takeuchi et al., 2005). Terpenes are known to be the major components of citrus essential oils, which contribute to the characteristic citrusy and woody notes (Cheong et al., 2012b). Included are major volatiles identified in the zest: limonene, a dominant terpene commonly reported in citrus fruits, and has the highest concentration of a volatile contained in the zest; germacrene D exhibits a woody spice aroma; geranyl acetate has an aroma described as green, waxy, and a floral rose with an oily, soapy, and citrus flavor; α-pinene presents fresh, sweet, and piney aromatic notes and its flavor is intense woody, piney, herbal, and spicy with slightly tropical nuances; β-myrcene; linalool is associated with lavender and blueberry characteristics, also described as having floral, sweet, and green aromas and the flavor of floral, waxy, aldehydic and woody; decanal has an aroma that is sweet, waxy, and

orange-peel like and a flavor of waxy, fatty, and citrusy with slight green melon nuance, and 1-octanol's aroma is waxy and mushroom-like with a green, citrus, and aldehydic flavor (The Good Scents Company, 2018).

There are 27 volatiles identified in the zest that have not been previously reported. These included methyl acetate, ethyl acetate, methyl alcohol, isopropyl alcohol, 3-pentanone, pseudolimonene, thunbergol, o-cymene, isoterpinolene, *(Z)*-2-pentenol, *(E)*-4,8-dimethylnona-1,3,7-triene, *(E)*-3-hexenol, perillene, dehydro-ρ-cymene, cosmene, *(E)*-2,8-ρ-mentha-dien-1-ol, γ-muurolene, *(Z)*-4-decenol, *(E)*-2-undecen-1-ol, α-calacorene, ρ-mentha-1-en-9-ol, 1-dodecanol, guaiol, isospathulenol, 8-hydroxylinalool, ethanol, and benzophenone. Volatiles identified for the first time in calamondin zest have been reported in the peel of several citrus fruits, such as in cosmene, which has been reported in the peel of Kaopan pummelo (Liu, Cheng, Zhang, Deng, Chen, & Xu 2012). In a study on volatile constituents of peel extracts of Redblush grapefruit (*Citrus paradisi*) and Pummelo (*Citrus grandis*) from Kenya, *(E)*-2,8,-ρ-mentha-dien-1-ol was found in both pummelo and Redblush grapefruit, and perillene was identified in Redblush grapefruit (Njoroge, Koaze, Karanja, & Sawamura 2005). Reports on analysis of peel extract volatiles *(Z)*-2-pentenol and γ-muurolene identified in the zest are reported in trace amounts in the peel extract of Australian finger lime (*Citrus australasica*; Delort & Jaquier, 2009). γ-muurolene has also been identified in the peel extract of the unripe shiikuwasha (*Citrus depressa hayata*), a citrus fruit similar to calamondin in size, shape, and flavor (Asikin et al., 2012). Methyl acetate has been reported in SPME analysis of the pink and white Malaysian pomelo peel

(Cheong et al., 2011). Results of SPME-GC-MS volatile analysis of the zest are reported here for the first time.

SPME was adopted to extract compounds at trace levels and to study the original volatile profile with minimum changes to the fresh juice and freshly grated zest. Limiting factors of the SPME method are its inability to extract compounds at certain polarities and heavy volatile compounds, thus analysis does not provide an extensive volatile profile. Conversely, the SPE method has an advantage of extracting a broad range of nonpolar to very polar compounds so integrating both extraction methods can provide a wide range of volatiles from the calamondin samples (Andrade-Eiroa et al., 2016b).

SPE Solvent Elution Comparison

SPE is a widely used method for the extraction, concentration, and fractionation of organic compounds from various types of samples (Andrade-Eiroa et al., 2016b). Optimal extraction of food aroma compounds is dependent on the sorbent used to target specific groups: silica gels (polar due to their hydroxyl groups), activated aluminas (polar), activated carbon (apolar), zeolites and polymers, such as polystyrene, polyacrylilc esters, PDMS and phenolic resins (Dziadas et al., 2011). SPE has been extensively reported in the analyses of wine where styrene-divinylbenzene (SDVB) sorbent, the type of sorbent used in this study, was tested along with dichloromethane for elution that resulted in ideal extraction and recoveries of terpenes, and satisfactory extraction of important wine volatiles such as phenols, vanillin derivatives, alphatic lactones, nor-isoprenoids, esters, and terpenols (Dziadas et al., 2011; López, Aznar, Cacho, & Ferreira 2002; Pineiro, Palma,

& Barroso 2004). SPE using a divinylbenzene sorbent (LiChrolut-EN) has also been reported in the study of blackberry juice that resulted in effective extraction of furaneol and less retention of pigments and other non-volatiles than HLB and C18 sorbents (Du & Qian, 2008). Utilizing a range of solvents for SPE sample elution and comparing their volatile extracts is a means to method optimization, as a majority of SPE protocols are optimized by trial-and-error (Andrade-Eiroa et al., 2016b). Due to the range of concentrations in which flavor compounds are present in a food sample, a fractionation using solvents of different polarities would be ideal (Dziadas et al., 2011).

Chromatographic comparison of SPE using LiChrolut EN and 30 mL mixed solvents at various ratios of dichloromethane and methanol all extracted three main compounds known to be in calamondin: limonene, linalool, and α-Terpineol. Concentrations of these compounds eluted in varying concentrations as evidenced by the peak size: the 100% methanol solution expressing the lowest concentration and the 95:5 dichloromethane:methanol solution expressing the highest concentration. Comparison of chromatographs indicated that 95:5 dichloromethane:methanol solution also yielded the most peaks, indicating the solvent ratio is ideal for volatile extraction. This suggests that in this proportion, dichloromethane's moderate polarity properly stabilizes with methanol's polarity, producing a balanced solvent for ideal volatile extraction, which included a wide range of volatiles with various polarity and molecular weights.

Compounds that are by-products of biological processes such as the breakdown of pigments or dehydration of sugars are identified as artifacts in GC-MS chromatograph. In

studies comparing flavor isolation methods of SAFE, SPME, and SDE, 5 components were identified in the SDE extract that were not identified in the SAFE and SPME extracts; these components formed may be due to the long-term influence of high temperature that the sample is exposed to during the SDE method (Majcher & Jeleń, 2009; Wieczorek, Majcher, & Jeleń 2020). Artifacts identified in this study, furfural and 5-hydroxymethylfurfural, have also been reported in analysis of honey in which it is a well-known artifact formed by heating (Rivellino et al., 2013). Artifacts present are also an indication of poor extraction due to solvent polarity not corresponding with target analytes. Another aspect to consider is the stability of the food sample in the solvent; when the sample is dissolved or solvated in a solvent (i.e., solvent extraction) chemical reactions take place that may chemically alter the sample and form derivatives (Maltese et al., 2009). Artifact formation leads to formation of new compounds, loss of activity of active components, and loss in total yield of important volatiles (Maltese, van der Kooy, & Verpoorte 2009). In this study, all solvent elutions except for the 95:5 dichloromethane:methanol solution have evidence of artifacts, again suggesting that this ratio is ideal for volatile extraction.

95:5 Dichloromethane:Methanol Solvent Elution – 30 mL vs 1 mL

Using 1 mL of solvent for elution is a common protocol of SPE that has been utilized in flavor isolation (Du & Qian, 2008). After confirming SPE with 95:5 dichloromethane:methanol solvent elution resulted in ideal volatile extraction, 1 mL elution with the solvent ratio was executed on the calamondin juice. Chromatograph analysis presented approximately eight less significant peaks and less concentration of

volatiles compared to the chromatograph of elution with 30 mL of solvent. This difference is due to the smaller volume used to eluate the sorbent, resulting in residual volatiles not desorbing from the solid-phase sorbent (Andrade-Eiroa et al., 2016a).

Therefore, using 30 mL of 95:5 dichloromethane:methanol solvent elution for SPE analysis method proved to be optimal for extraction of volatiles in the calamondin samples. To date, reported volatile isolation methods used on calamondin are SPME, solvent extraction, distillation, and cold press extraction (Chen et al., 2013; Cheong et al., 2012a; Cheong et al., 2012b; Cuevas-Glory et al., 2009; Moshonas et al., 1996; Nisperos-Carriedo et al., 1992; Takeuchi et al., 2005; Yamamoto et al., 2012; Yo et al., 2004). SPE method for calamondin volatile isolation is reported here for the first time.

Volatile Composition in Calamondin Juice and Peel using SPE-GC-MS

SPE-GC-MS Volatiles Identified in Calamondin Juice

A total of 75 volatiles were identified in the juice by SPE-GC-MS with limonene, *(Z)*-3-hexenol, α-terpineol, α-cadinol, limonen-1,2-diol, *(Z)*-8-hydroxylinalool, cryptomeridiol, hexadecanoic acid, 4-hydroxy-benzeneethanol, stearic acid, and linoleic acid being the most abundant compounds. Previous reports state that limonene is the most abundant of all the volatiles, followed by germacrene D, β-myrcene, linalool, α-terpineol, and terpinen-4-ol (Cheong et al., 2012b; Takeuchi et al., 2005; Yamamoto et al., 2012; Yo et al., 2004).

38 volatiles identified in the juice are reported here for the first time: prenol, isomenthone, fenchol, menthol, benzeneacetaldehyde, 2-furanmethanol, isopiperitenol, α-

muurolene, selina-4(15),7(11)-diene, 3,4-dimethyl-benzaldehyde, 3-methyl-2-butenoic acid, isopropyl dodecanoate, hexanoic acid, benzyl alcohol, (*Z*)-ρ-mentha-1(7),8-dien-2-ol, phenylethyl alcohol, ρ-menth-1-en-9-ol, dodecanol, 2-pyrrolidinone, viridiflorol, hinesol, α-cadinol, 2-methoxy-4-vinylphenol, intermedeol, 4-methyl-5-thiazoleethanol, (*Z*)-8-hydroxylinalool, (*E*)-isoeugenol, coumaran, benzophenone, 3-hydroxy-β-damascone, vanillin, (*E*)-8-hydroxygeraniol, cryptomeridiol, 2,6-dimethoxy-4-propenylphenol, syringylaldehyde, α-copaen-11-ol, 4-hydroxy-benzeneethanol, and coniferyl alcohol. Several polar volatiles with high boiling points, such as (*Z*)-8-hydroxylinalool, (*E*)-isoeugenol, 3-hydroxy-β-damascone, vanillin, (*E*)-8-hydroxygeraniol, cryptomeridiol, 2,6-dimethoxy-4-propenylphenol, syringylaldehyde, α-copaen-11-ol, 4-hydroxy-benzeneethanol, and coniferyl alcohol can only be isolated by SPE or solvent extraction, while SPME could not extract them. (*E*)-isoeugenol has a sweet, spicy, and floral aroma; syringylaldehyde has a chocolate, woody aroma, and a sweet, cocoa, creamy, and dairy-like flavor (The Good Scents Company, 2018). Vanillin is a well-known component of fruits and fruit juices, such as mango, elderberry juice, blueberries, orange juice, strawberries, passion fruit juice, and lychee (Goodner, Jella, & Rouseff, 2000). Reports indicate that mass spectral identification confirms the presence of vanillin in grapefruit, lemon, lime, and tangerine juices (Goodner et al., 2000). (*Z*)-8-hydroxylinalool has been reported a component of citrus flowers of the same germplasm (Zhang et al., 2020).

Volatile compounds identified for the first time in juice are (*Z*)-ρ-mentha-2,8-dien-1-ol and decanol, which have been previously reported in the peel oil; and decanol, γ-muurolene, α-amorphene, δ-terpineol which have been previously reported in the leaf oil (Cuevas-Glory et al., 2009; Takeuchi et al., 2005).

SPE-GC-MS Volatiles Identified in Calamondin Peel

A total of 101 volatiles were identified in the peel by SPE-GC-MS with limonene, (*Z*)-3-hexenol, linalool, 1-octanol, α-terpineol, 8-hydroxylinalool, and hexadecanoic acid being the most abundant compounds. Previous reports on calamondin peel identify major components as limonene, myrcene, germacrene D, β-pinene, linalool, and α-terpineol. Although these reports specify the fruit is in the mature stage, which may impart a different aroma profile than the calamondin in its green-mature stage that was analyzed in this study (Chen et al., 2013; Cheong et al., 2012a; Cuevas-Glory et al., 2009; Moshonas et al., 1996; Nisperos-Carriedo et al., 1992; Takeuchi et al., 2005).

39 volatiles identified in the peel are reported here for the first time: 1-penten-3-ol, (*E*)-2-hexenal, 1-pentanol, 3-penten-1-ol, (*Z*)-2-hexen-1-ol, (*E,E*)-2,4-hexadienal, (*E*)-2-hexenol, (*E*)-2-octenal, isopinocampheol, (*E*)-4-decenal, (*E*)-sabinene hydrate, (*E*)-2-octen-1-ol, α-santalene, isogermacrene D, neodihydrocarveol, *(Z)*-isopiperitenol, *(E)*-isopiperitenol, 2-decen-1-ol, *(E)*-ρ-mentha-1(7),8-dien-2-ol, *(E,E)*-2,6-dimethyl-3,5,7-octatriene-2-ol, hexanoic acid, benzyl alcohol, *(Z)*-ρ-mentha-1(7),8-dien-2-ol, phenylethyl alcohol, ρ-menth-1-en-9-ol, dodecanol, heptanoic acid, phenol, γ-decalactone, 2,6-dimethyl-1,7-octadiene-3,6-diol, 2-methoxy-4-vinylphenol, jasmine lactone, 8-

hydroxylinalool, isoelemicin, indole, vanillin, 8-hydroxygeraniol, perillic acid, and cryptomeridiol. Studies of the citrus peel have reported similar volatiles. Indole has been identified in pink and white Malyasian pomelo (*Citrus grandis*) peel and (*E*)-2-hexenol has been identified in the white pomelo (Cheong et al., 2011). A study comparing the peel volatiles of Mangshanyegan (*Citrus nobilis* Lauriro), a wild mandarin type orange, to four other citrus species: Kaopan pummelo (*Citrus grandis*), Eureka lemon (*Citrus limon*), Huangyanbendizao tangerine (*Citrus reticulata*), and Seike navel orange (*Citrus sinensis*), all contained similar volatiles identified in this study: 8-hydroxylinalool – Mangshanyegan, Eureka lemon; 1-penten-3-ol – Mangshanyega, Seike navel orange, Huangyanbendizao tangerine; and (*E*)-2-hexenol known for its fruity, green, unripe banana aroma, and leafy, fresh, and juicy flavor is reported in the peel of Mangshanyega orange, Kaopan pummelo, Seike navel orange, and Huangyanbendizao tangerine (The Good Scents Company, 2018; Liu et al., 2012). In addition, those heavy and polar volatiles with high boiling points, such as phenol, γ-decalactone, 2,6-dimethyl-1,7-octadiene-3,6-diol, 2-methoxy-4-vinylphenol, jasmine lactone, 8-hydroxylinalool, indole, vanillin, 8-hydroxygeraniol, could not be extracted by SPME, but isolation was possible based on polarity such as solvent extraction and SPE method.

Also identified for the first time in the peel are hexanal, 3-carene, *(E)*-linalool oxide, 2-ethylhexanol, benzaldehyde, nonyl acetate, *(Z)*-dihydrocarvone, *(E)*-dihydrocarvone, *(E)*-ρ-mentha-2,8-dien-1-ol, d-carvone, piperitenone, 2,6-dimethyl-3,7-octadiene-2,6-diol, *(E,E)*-2,4-decadienol, limonen-10-ol have been previously identified in

juice; β-copaene, *(E)*-dihydrocarvone and decanol have been previously identified in peel oil; and β-copaene**,** *(E)*-ρ-mentha-2,8-dien-1-ol, γ-muurolene, and decanol have been previously identified in the leaf oil (Chen et al., 2013; Cheong et al., 2012a; Cheong et al., 2012b; Cuevas-Glory et al., 2009; Takeuchi et al., 2005; Yamamoto et al., 2012).

Previous reports of isolation methods on calamondin peel are by SPME, solvent extraction, distillation, and cold-pressing (Chen et al., 2013; Cheong et al., 2012a; Cuevas-Glory et al., 2009; Moshonas et al., 1996; Takeuchi et al., 2005). Both SPE analysis of the juice and peel have resulted in extraction of more than 70 compounds not previously reported. This evidence indicates SPE extraction abilities for flavor compounds.

Seasonal Variation of Volatiles Identified in Calamondin Peel and Juice

Volatiles are second metabolites that can be impacted by endogenous factors (e.g., genotypes and species) and exogenous factors (e.g., climates and geography). Harvest date is an exogenous factor and volatiles in both calamondin peel and juice are impacted by harvest date. Comparison of calamondin volatile profiles from spring harvest and summer harvest have variations in compounds and their intensities. Literature indicates similar outcomes, such as in a study of volatile comparison of blood orange harvested at three different times (Tounsi, Mhamdi, Kchouk, & Marzouk 2010). Terpenic compounds and their variation during seasonal cycles has been irregular, which can be explained by monoterpene synthases producing more than one compound, particularly limonene's metabolic role of converting into linalool, linalyl acetate, ρ-cymene, etc. (Tounsi et al., 2010). Pre-harvest factors such as sunlight, water availability, fertilization, and chemical

applications affect crop growth, and can affect internal quality characteristics of the harvested product, including flavor (El Hadi, Zhang, Wu, Zhou, & Tao, 2013). It has been reported that harvest date is linked to environmental parameters such as temperature, relative humidity, and total duration exposure to sun and wind patterns that influence metabolism, catabolism, and biosynthesis of volatile compounds (Ellouze et al., 2012).

Quantitative Descriptive Analysis

The only known QDA study of calamondin has been on the aroma of peel extracts which used floral, fatty, fruity, green, juicy, mandarin-like, peely, woody, and sweet as sensory descriptors (Cheong et al., 2012a). This study used the same descriptors for QDA of calamondin with the addition of lime, piney, grapefruit, and acidic, as well as sourness, astringent, bitter, and salivating for the taste of the juice.

For this sensory analysis, references were developed for the 13 descriptors, in which there is only one publication on the sensory analysis of the peel extract (Cheong et al., 2012a). Reference materials were used to establish a common vocabulary for various aromas and flavors. A reference standard can be any chemical or natural material that adequately represents the particular characteristic described (Krasner, 1995). Using examples can increase a panelist's understanding of important attributes; however, examples are less singular in terms of flavor perception than references (Lawless & Civille, 2013). Examples have a prominent component that illustrates a specific attribute, but other attributes can be confusing to panelists (Lawless & Civille, 2013). In other words, singular references are preferred as long as they are practical, while examples are less restricted

(Lawless & Civille, 2013). In addition, using chemical as reference has advantages of keeping consistent composition for the attribute and can easily be reproduced by others.

Quantitative descriptive analysis concluded that the smell of the calamondin zest exhibited peely as being the most intense attribute, in which the only sensory publication to date on calamondin also states as its highest ranked attribute, although this sensory analysis was done on orange-mature calamondin extract (Cheong et al., 2012a). Other dominant attributes of the peel are fresh, fatty, waxy, green, that can be attributed to terpenes contained in the peel. Overall, calamondin peel, like other citrus peels such as lemon, lime, and orange, has a very rich aroma.

Most intense attributes of the aroma of the juice are juicy, acidic, mandarin, fresh, green, and grapefruit, and most intense attributes of the taste of the juice are sourness, salivating, and astringent. Reported here for the first time are aroma and taste sensory analysis of calamondin juice. Overall, calamondin juice is very sour, and could be used as a substitution for lemon or lime.

Link Sensory to Chemical Analysis

Sensory data correlates to chemical data as the compounds contained in the dominant attributes of the peel and juice have been identified in the samples. The peely attribute consists of the compounds octanal and decanal; fresh consists of acetaldehyde; fatty consists of octanal; and green consists of cis-3-hexenol, all of which have been identified in the peel and juice. The knowledge of the link between sensory attributes and volatiles has been used to formulate chemical reference for QDA of this research.

The sourness attribute of the taste of the juice is contributed to the low pH value and the salivating attribute is contributed to the high amount of citric acid contained in calamondin's juice. TA (equivalent to citric acid) for calamondin is extremely high, compared to other fruit juice such as orange (Cheong et al., 2012b; Sinclair, Bartholomew, & Ramsey, 1945).

CHAPTER VI

CONCLUSION

The volatiles in green-immature calamondin peel and juice were extracted and analyzed, with a comparison between the most common used method (SPME) and less commonly used (SPE). SPE method was developed and optimized. Development of solid-phase extraction (SPE) method using LiChrolut EN sorbent and dichloromethane: methanol (95:5) elute can specifically extract heavy, polar volatiles. SPE method identified 38 volatiles from the juice and 39 volatiles from the peel that have not been previously reported, and harvest date has impact on volatile profiles in quality and quantity. The results add new knowledge to literature. Sensory analysis was conducted with 12 trained panelists. Descriptors were created with chemical references developed for each attribute. The flavor profiles of the calamondin juice and peel were identified and expressed in 13 attributes. PCA indicated that the zest smelled highly in green, waxy, peely, fatty, fresh, woody, piney, and lime notes; the juice smelled highly of fruity, juicy, and mandarin notes, and the juice taste had a grapefruit-like aroma. A major limitation in this study is that it mainly focused on volatile profile analysis, whereas including an aroma profile analysis with GC-MS/O will give more insight about volatiles contributing to calamondin aroma. However, this study may be effective in SPE method development for food analysis of juice and peel and application towards developing a flavor profile.

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APPENDIX A

Institutional Review Board Approval Letter

TWU
TEXAS WOMAN'S UNIVERSITY
DENTON DALLAS HOUSTON

Institutional Review Board
Office of Research and Sponsored Programs
P.O. Box 425619, Denton, TX 76204-5619
940-898-3378
email: IRB@twu.edu
http://www.twu.edu/irb.html

DATE: February 6, 2018

TO: Dr. Xiaofen Du
Nutrition & Food Sciences

FROM: Institutional Review Board (IRB) - Denton

Re: Exemption for Exploring Freshness Flavor or Calamondin (Citrus Microcarpa) Peel and Juice by Sensory and Instrumental Analysis (Protocol #: 19951)

The above referenced study has been reviewed by the TWU IRB (operating under FWA00000178) and was determined to be exempt from further review.

If applicable, agency approval letters must be submitted to the IRB upon receipt PRIOR to any data collection at that agency. Because a signed consent form is not required for exempt studies, the filing of signatures of participants with the TWU IRB is not necessary.

Although your protocol has been exempted from further IRB review and your protocol file has been closed, any modifications to this study must be submitted for review to the IRB using the Modification Request Form. Additionally, the IRB must be notified immediately of any adverse events or unanticipated problems. All forms are located on the IRB website. If you have any questions, please contact the TWU IRB.

cc. Dr. Shane Broughton, Nutrition & Food Sciences

APPENDIX B

Institutional Review Board Consent Form

TEXAS WOMAN'S UNIVERSITY
CONSENT TO PARTICIPATE IN RESEARCH

Title: Exploring Freshness Flavor of Calamondin (Citrus microcarpa) Peel and Juice by Sensory and Instrumental Analysis

Investigator: Xiaofen Du, PhD.....xdu@twu.edu [redacted]

Explanation and Purpose of the Research

You are being asked to participate in a research study for Xiaofen Du at Texas Woman's University. The purpose of this research is to discover and characterize aroma-active and freshness perception associated molecules in calamondin peel and fruit and the possible chemical mechanism of freshness perception. To fulfil this goal, the specific research aims will be: I. Quantitative descriptive analysis of calamondin fruit; II. Isolation and identification of potential aroma-active and flavor-modifying molecules in calamondin fruit; III. Gas chromatography- Olfactory analysis screening molecules associated with freshness perception; and IV. Screening and characterization of the freshness effects of the target molecules by taste.

Description of Procedures

In order to be a participant in this study, you must be at least 18 years of age or older and consume citrus fruits regularly. The overall procedure will be:

Fresh calamondin fruit will be picked up from a backyard in the Dallas area. The fresh fruit will be used for sensory evaluation. Quantitative Descriptive Analysis (QDA) will be carried out in the sensory lab at Texas Woman's University. Ten panelists will be recruited from students and staff at TWU and trained. Using calamondin fruit, the panel will develop descriptive lexicons, along with definitions and references. The panel will then be trained over several sessions to practice rating the intensity of the attributes in each profile. The intensity of each attribute will be evaluated across the products on an unstructured, 10-cm line scale. All products will be served in 2 oz plastic portion cups covered with a plastic lid. The tests will be conducted in isolated booths illuminated with incandescent lighting. Judges will rinse between samples with bottled spring water. Each product will be evaluated in duplicate. All instructions, scale presentations, and data collection will be carried out manually.

Potential Risks

Allergens, as with all food products, may be a concern for consumers allergic to fresh calamondin fruit. All participants will be verbally screened for allergens prior to participating in the taste-testing. The procedures and fruits in this experiment post no additional risks compared to foods normally eaten by consumers.

There is potential risk of loss of confidentiality in all email and downloading. Confidentiality will be protected to the extent that is allowed by law.

The researchers will try to prevent any problem that could happen because of this research. You should let the researchers know at once if there is a problem with food allergic and they will help you. However, TWU does not provide medical services or financial assistance for injuries that might happen because you are taking part in this research.

_____
Initials

Participation and Benefits

Your involvement in this study is completely voluntary and you may withdraw from the study at any time. Following the completion of the study you will receive a $ 40 gift card for your participation. If you would like to know the results of this study we will mail them to you.*

Questions Regarding the Study

You will be given a copy of this signed and dated consent form to keep. If you have any questions about the research study you should ask the researchers; their phone numbers are at the top of this form. If you have questions about your rights as a participant in this research or the way this study has been conducted, you may contact the Texas Woman's University Office of Research and Sponsored Programs at 940-898-3378 or via e-mail at IRB@twu.edu.

_____ _____

Signature of Participant Date

*If you would like to know the results of this study tell us where you want them to be sent:

Email: _____
or
Address:

_____

_____

_____

APPENDIX C

Sensory Ballot

WELCOME TO THE CALAMONDIN PANEL!

You are participating in a study to evaluate specific flavor components in calamondin fruit.

- **If you know you are allergic to calamondin fruit, please withdraw from the panel.**
- **If you are or think you are pregnant, if you are nursing, or if you are immune compromised, please withdraw from the panel.**

The sensory evaluation includes two parts: SMELL and TASTE

- **Smell the sample and rate ALL the attributes. Make sure you are taking the correct sample.**
- **Taste the sample and rate ALL attributes. Make sure you are tasting the correct sample.**
- **Keep calibrating yourself with the standards during the panel.**
- **Please use the comment line. Comments are very useful.**

THANK YOU!

SMELL – CALAMONDIN ZEST

Fresh

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Fatty

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Green

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Peely

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Waxy

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Fruity

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Juicy

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Lime

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Piney

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Floral

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Woody

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Mandarin

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Grapefruit

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Acidic

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Sweet

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Overall Comments _____

Sample 1:0

SMELL – CALAMONDIN JUICE

Fresh

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Fatty

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Green

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Peely

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Waxy

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Fruity

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Juicy

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Lime

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Piney

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Floral

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Woody

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Mandarin

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Grapefruit

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Acidic

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Sweet

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Overall Comments _____

Sample 1:2

TASTE – CALAMONDIN JUICE

Fresh

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Fatty

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Green

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Peely

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Waxy

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Fruity

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Juicy

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Lime

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Piney

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Floral

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Woody

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Mandarin

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Grapefruit

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Sourness

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Sweet

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Astringent

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Bitter

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Salivating

0___1___2___3___4___5___6___7___8___9___10 _____
Low medium high additional comments

Overall Comments _____

APPENDIX D

Demographic Form

Please give a little information about your calamondin experience

Question # 1.

How often do you **purchase** citrus fruit / citrus juice?

- Once a week or more often
- Every 2-4 weeks
- Less than once a month
- Never

Question # 2.

How often do you **eat** citrus fruit / citrus juice?

- Several times a week
- 1-4 times per month
- Only occasionally

Question # 3.

Which type of citrus fruit / citrus juice do you consume most frequently?

- Orange
- Mandarin / Tangerine
- Grapefruit
- Lemon
- Lime
- Others_____

Question # 4.

If you are a citrus fruit buyer/eater, which type of product do you prefer?

- Fresh (raw)
- Juice
- Canned
- All of them
- N/A (I do not consume citrus on a regular basis)

Question # 5.

What is your age group?

- <25
- 25-35
- 36-45
- 46-55
- 56-65
- >65

Question # 6.

If you have any general comment about citrus fruit and/or those you have tasted today, please write it here.

_____

_____